TRANSFORMATION OF 'NOVA' TANGELO AND 'ROHDE RED VALENCIA' SWEET ORANGE WITH THE COAT PROTEIN GENE OF CITRUS TRISTEZA CLOSTEROVIRUS

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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There has been considerable interest in using plant molecular biology for crop improvement. The technology to isolate and clone a specific gene is commonly utilized today. The methodology to insert these genes has been well established for many crop species. One of these methods is the use of PEG-mediated direct DNA uptake. This method relies on the availability of a reliable protoplast isolation and plant regeneration system, which is available for *Citrus*.

Protoplasts isolated from ovule-derived callus of 'Nova' tangelo ('Clementine' mandarin (*Citrus reticulata* Blanco) X 'Orlando' tangelo (*C. reticulata* X *C. paradisi* Macf)) and 'Rohde Red Valencia' sweet orange (*C. sinensis* L(Osbeck)) were transformed by PEG-mediated direct DNA uptake using plasmids containing the coat protein gene of citrus tristeza virus (CTV). Three transgenic 'Nova' tangelo plants were obtained using plasmid pMON10098 containing the

coat protein gene of the Florida mild isolate T30. Twenty-two transgenic 'Rohde Red Valencia' sweet orange plants were obtained using plasmid pBPFΩ7 containing the coat protein gene of the Florida severe isolate T36. Transformants were selected by PCR analysis or dot blot hybridization. Confirmation of transformation was by Southern hybridization and western immunoblot analysis. These transgenic plants will now be budded onto sour orange rootstock and subjected to challenge inoculation with severe isolates of citrus tristeza virus in the greenhouse, to determine if they are resistant to CTV.

CHAPTER 1

INTRODUCTION

There is considerable interest in using plant molecular biology techniques for crop improvement. The technology to isolate and clone a specific gene is commonly utilized today. The number of genes and proteins isolated and sequenced is in the thousands, and they are cataloged in several databases. Latterich and Croy (1993) have published a partial listing of these databases and many of the genes and proteins isolated.

Along with the advances in gene and protein isolation and characterization has been an increased interest in using this knowledge to clone and insert these genes into plants. Transgenic plants have been engineered with several purposes in mind, including development of new and more efficient transformation methods, for the basic study of a specific gene, and for crop improvement for a specific purpose (Kung, 1993). The first transgenic plants were produced in the early 1980s. Murai et al. (1983) transferred the β-Phaseolin gene from bean to sunflower and tobacco plants using *Agrobacterium tumefaciens*. At around the same time, several transgenic tobacco plants transformed using *Agrobacterium tumefaciens* vectors and expressing foreign genes were reported (Horsch et al.,1984; De Block et al., 1984).

These first transformation reports, as well as many that followed, utilized the *Agrobacterium tumefaciens* vector system. However a number of alternative methods have been developed. These include direct DNA uptake, electroporation, particle bombardment, and microinjection. These methods are discussed in Chapter 2.

Citrus tristeza virus (CTV) is the most economically important viral pathogen of citrus (Lee and Rocha-Pena, 1992). This member of the closterovirus group has a flexous rod shaped particle and a positive-sense, single stranded RNA genome with a molecular weight of approximately 6.5×10^6. The genome of CTV contains 19,296 nucleotides constituting a single messenger- sense RNA, making it the largest single-stranded, plus-sense RNA plant virus known (Karasev et al., 1995). The coat protein gene was first identified and sequenced by Sekiya et al. (1991). Of the nearly 20 kb in the genome, the 7292 nucleotides at the 3' end were sequenced and eight open reading frames (ORFs) were identified, including the coat protein gene, by Pappu et al. (1994). The remaining genome was sequenced by Karasev et al. (1995), and it was thus determined that the complete genome encodes 12 ORFs, and potentially codes for at least 17 gene products. CTV is transmitted in a semipersistent manner by several species of aphids, most notably *Aphis gossypii* and *Toxoptera citricida*. *T. citricida* is a much more effective vector than is *A. gossypii* (Lee and Rocha-Pena, 1992).

Citrus tristeza virus exists as a number of strains with different biological activities. Mild isolates which show little to no symptoms are often found in most citrus plantings. Mild strains will cause a slight stem-pitting, little to no vein clearing, and flecking in the indicator

plant, Mexican Lime (*Citrus aurantifolia*). The more serious seedling yellows symptoms can be found in seedlings of sour orange (*C. aurantium*), lemon (*C. limon*), and grapefruit (*C. paradisi*). Seedling yellows is characterized by a severe chlorosis and dwarfing. Stem pitting can occur in both sweet orange and grapefruit. These trees will be stunted and often chlorotic, with pitting under the bark of the stem and branches. The stunted trees will often show pronounced longitudinal ridges or depressions running up and down the trunk. Fruit size and productivity is greatly reduced. One of the more severe form of the tristeza disease is quick decline. This is a disease of sweet orange grafted on sour orange rootstock. With quick decline the leaves turn yellow or golden in color, wilt and then fall from the tree, leaving only the fruit hanging from the tree. The tree quickly dies. Often the bud union will have an overgrowth immediately above it (Lee and Rocha-Pena, 1992). Plants exhibiting quick decline symptoms often succumb to the disease in as little as three to six weeks.

Since Beachy and his colleagues were able to transform tobacco with the coat protein gene of tobacco mosaic virus (TMV) and obtain resistance to TMV (Powell-Abel et al., 1986; Beachy et al., 1987) there has been considerable interest in using this technology to develop plants resistant to other viruses (See chapter 2 for a review of this work). The purpose of the present study was to apply this technology to insert the coat protein gene from CTV into citrus scion cultivars in an attempt to engineer virus resistant plants. If successful, this could be a significant step in eliminating one of the major disease problems in

Citrus, and could lead to the possibility of once again using the CTV susceptible rootstock sour orange in areas where CTV is found.

CHAPTER 2

LITERATURE REVIEW

Methods of Transformation

Agrobacterium tumefaciens-mediated Transformation

Agrobacterium tumefaciens is the pathogen that causes crown gall disease in many plant species. The infection cycle of *Agrobacterium* is very complex, involving a number of chemical signals emitted by both the host and the pathogen. Infection occurs when the bacteria are attracted to a number of chemicals secreted by wounded plant cells (Gelvin, 1993). The virulent strains of *A. tumefaciens* contain large plasmids which are responsible for the DNA transfer that subsequently causes the gall formation. These plasmids have been termed tumor-inducing or Ti plasmids (Schell et al., 1979; Chilton et al., 1980). Among the genes residing in the Ti plasmids are the genes for opine synthesis in infected plants, and opine catabolism in the *Agrobacterium* (Bomhoff et al., 1976; Genotello et al., 1977). Early studies had shown a genetic linkage between oncogenicity and opine metabolism (Lippincott et al., 1973; Genetello et al., 1977). This led to the hypothesis that somehow the Ti plasmid was involved in some sort of DNA transfer mechanism from the bacterium to the plant. This DNA transfer mechanism was first shown to occur by Chilton et al (1977) using hybridization experiments. This same group later showed

transcription of a part of this T-DNA (transferred DNA) in the crown gall cells (Drummond et al., 1977).

The T-DNA covalently integrates into plant nuclear DNA, where genes encoded by the T-DNA direct the synthesis of auxins and cytokinins, as well as the low molecular weight opines. Opines are secreted from the tumorous cells and can be used as a carbon, and sometimes, nitrogen source by the *Agrobacterium*. Some of these opines also induce the conjugal transfer of the Ti plasmid between *Agrobacterium* cells (Gelvin, 1993). Thomashow et al. (1980 a,b) demonstrated that a majority to all of the T-DNA is integrated into the plant DNA and that the T-DNA can be inserted in more than one site in the plant genome. The excision of the Ti plasmid and its subsequent transfer from the *Agrobacterium* to plant cells is controlled by a group of genes on the Ti plasmid called the *vir* genes. These *vir* genes are induced by a group of phenolic compounds produced by the plant (Garfinkel and Nester, 1980; Klee et al., 1982; Hooykaas et al., 1984; Lundquist et al., 1984; Gelvin, 1993).

The T-DNA contains specific DNA sequences called T-DNA borders which have been shown by mutational analysis to be essential for tumorigenesis. These T-DNA borders are made up of 25 bp highly conserved DNA sequences in a directly repeated orientation (Schell, et al., 1979; Lemmers et al, 1980; Thomashow et al., 1980a,b; Zambryski et al., 1983; Bevan, 1984). The mechanisms by which the DNA crosses the plant cell wall, crosses through the cytoplasm, enters into the nucleus, and becomes integrated into the plant DNA, are still unknown.

It was these T-DNA properties that led researchers to investigate the use of *Agrobacterium* and its Ti plasmid as a tool for genetic

engineering. In order for this to be possible, two major modifications were found to be necessary. First, the non-essential sequences (those not involved in T-DNA transfer) of the T-DNA must be replaced with the foreign genes of interest. Secondly, the tumor inducing capabilities of the T-DNA need to be inactivated without inactivating its DNA transfer capabilities (Van Montagu and Schell, 1982). In addition, the large size of the Ti plasmid (>200 kb) makes it necessary to produce a series of autonomously replicating or integrative vectors to use in gene transfer. These integrative vectors are constructed in such a way that they recombine specifically with a resistant Ti plasmid in *A. tumefaciens* which has been disarmed. An example of a disarmed *A. tumefaciens* Ti plasmid is pGV3850, a Ti plasmid mutant in which all the oncogenic functions have been deleted and replaced by pBR322. (The plasmid pBR322 was developed for use in a multipurpose cloning system prior to the identification of the Ti plasmids of *Agrobacterium* (Bolivar et al., 1977). This plasmid (pBR322) is a relaxed replicating plasmid that is sensitive to colicin E1 and also carries resistance to the antibiotics ampicillin and tetracycline. Plasmid pBR322 also contains five unique restriction sites- BamHI, EcoRI, HindIII, PstI, and SalI. The resulting pGV3850 plasmid is still capable of mediating efficient transfer and stabilization of its truncated T-DNA into infected plant cells, but its integration and expression does not interfere with normal plant function (Zambryski et al., 1983; Rogers et al., 1987). At a minimum, the chimeric genes that are integrated generally include a promoter such as the cauliflower mosaic virus gene VI promoter (Comai et al., 1983; Potrykus et al., 1985a,b) or the nopaline synthase promoter (de Block et al., 1984; Hain et al., 1985), as well as

a prokaryotic antibiotic resistance gene for antibiotics such as kanamycin, streptomycin, spectinomycin or chloramphenicol (Comai et al., 1983; Paskowski et al., 1984; Hain et al., 1985; Potrykus et al., 1985; Rogers et al., 1988). Generally other genes including a selectable marker such as kanamycin (NPTII gene), hygromycin (hph gene), or bialophos (bar gene) and/or reporter genes such as β-glucuronidase (uidA gene), or chloramphenicol acetyl transferase (cat gene) are also included for selection of transformed plants or cells.

Many of the plasmids constructed today are integrative intermediary plasmids that contain pBR322 sequences. Autonomously replicating vectors are constructed similarly to integrative vectors but contain an additional wide host range function and thus do not require a co-integrate formation for stable maintenance in *Agrobacterium* and subsequent transfer to plant cells (Hoekema et al., 1983; Bevan, 1984; Rogers et al., 1988).

Direct Gene Transfer Using Particle Bombardment

One of the most popular methods for gene transfer today is particle acceleration (bombardment). In this method, the DNA to be transferred is carried through the cell wall and into the cytoplasm on the surface of small (0.5 to 5 μm) metal (usually tungsten or gold) particles which have been accelerated to speeds of one to several meters per second (Klein et al, 1987). These particles are capable of penetrating up to several layers of cells and allow for the transformation of cells within tissue explants. This method is very popular for the transformation of many cereals in which protoplast culture is difficult, as well as dicots which can be recalcitrant to *Agrobacterium* infection.

Particle bombardment has several advantages over *Agrobacterium*-mediated transformation. First, nonhosts of *Agrobacterium*, such as monocots, can be transformed. Second, plasmid construction does not require insertion of the sequences essential for T-DNA replication and transfer in *Agrobacterium*. Third, the introduction of multiple plasmids, referred to as cotransformation, is possible with particle bombardment. Fourth, false positives resulting from growth of the *Agrobacterium* in host cells are eliminated. Finally, the transformation protocols are simplified by eliminating the complex bacteria-plant interrelationships present in *Agrobacterium* systems (Gray and Finer, 1993).

Virtually every type of cell or tissue has been used as a target for gene transfer by particle bombardment (See Table 2-1). Particle bombardment has been especially valuable for the transient assay of gene constructs in specialized cells or tissues (Goldfarb et al., 1991; Lee et al., 1991a; Seki et al., 1991). On the negative side, plants regenerated from bombarded plant tissues are usually chimeric in terms of introduced foreign genes due to random bombardment of a small number of cells in a multiple cell system (Sanford, 1990). This requires an additional selection using selectable markers to sort out transformants that are stabilized in their progenies (McCabe et al., 1988; Finer and McMullen, 1990; Fromm et al., 1990; Gordon-Kamm et al., 1990; Tomes et al., 1990; Christou et al., 1991; McCown et al., 1991; Lowe et al., 1995). In addition, the success of particle bombardment transformation to produce stable transgenic plants is partly dependent on having an efficient multiple shoot regeneration or embryogenic system. Further, the failure

Table 2-1: Types of cells or tissues used for particle bombardment, and whether or not transgenic plants were obtained.

Tissue used	Plant Species	Transgenic plants obtained	References
Suspension	Barley	no	Mendel et al., 1989
	Corn	yes	Gordon-Kamm et al., 1990
	Rice	no	Wang et al., 1988
	wheat	no	Wang et al., 1988
	Papaya	yes	Fitch et al., 1990
	Tobacco	no	Iida et al., 1990
	Cotton	no	Finer and McMullen, 1990
	Grape	no	Hebert, et al., 1993
Callus	Corn	yes	Fromm et al., 1990
	Rice	no	Oard et al., 1990
	Wheat	no	Daniell et al., 1991
	Peach	no	Ye et al., 1994
Embryos	Black Spruce	no	Bommineni et al., 1994
	Barley	no	Kartha et al., 1989
	Corn	no	Klein et al., 1989
	Pearl Millet	no	Taylor and Vasil, 1991
	Wheat	no	Lonsdale et al., 1990
	Rice	yes	Christou et al., 1991
	Peach	no	Ye et al., 1994
Leaf	Tobacco	yes	Tomes et al., 1990
	Wheat	no	Daniell et al., 1991
	Peach	no	Ye et al., 1994
	Sugarcane	no	Gallo-Meagher and Irvine, 1993
Meristems	Soybean	yes	McCabe et al., 1988
	Soybean	Yes	Christou et al., 1989
Nodules/Stems	Poplar	yes	McCown et al., 1991

to regenerate transgenic plants after particle bombardment of either embryogenic suspensions or immature scutelli may be due to the limited amount of DNA carried into the cells by particles or in the efficiency with which the DNA disassociates from the particle. As a result, there is a low potential for integration even in cells containing the particles. This low frequency has to be multiplied by the low frequency of competent cells. Thus the production of transgenics by particle bombardment can be expected at a low frequency (Potrykus, 1990).

The first report of microprojectile bombardment was made using a gunpowder-based acceleration system to propel tungsten particles carrying DNA through an evacuated chamber and into the cells (Klein et al., 1987). Later modifications were made to optimize the gun parameters and evaluation of gene expression (Russell et al., 1992; Morrish et al., 1993). Modifications of the gunpowder version of particle bombardment system used high pressure helium as a means to accelerate the microprojectiles (Russell-Kikkert, 1993). This design is commercially available from DuPont. A second design was a homemade version based on the DuPont airgun which utilized accelerated tungsten particles to transform monocot cells (Oard et al., 1990; Oard, 1993). A third concept utilized a stream of helium to deliver DNA (Takeuchi et al., 1992). This concept was further refined to become the commercially available particle inflow gun (PIG) (Finer et al., 1992).

Particle bombardment has made it possible to transfer foreign DNA into organelles. Daniell et al. (1990) reported on the introduction and transient expression of foreign genes in suspension cell-derived chloroplasts of tobacco. Daniell et al. (1991) also reported the

successful transient expression of β-glucuronidase in chloroplasts isolated from wheat leaf and callus and transformed using particle bombardment. Stable gene replacement at a very low frequency (a total of five transformants from 245 particle bombardment events) has been reported for tobacco (Svab et al., 1990; Staub and Maliga, 1992).

Gene Transfer By Direct DNA Uptake

In species with a reliable protoplast regeneration system, direct gene transfer by either chemical (polyethylene glycol (PEG)- mediated), or electroporation, or a combination of the two techniques has been quite popular. Protoplasts are in principle ideal cells for the delivery of DNA and selection of transgenic events. Removal of the cell wall eliminates one of the major barriers to the delivery of DNA. Further, the efficiency for recovery of transgenic events is higher because cross feeding and chimerism between transgenic and wild-type cells is minimized in comparison to transformation systems based on multicellular tissues (Songstad et al., 1995). Because plants regenerated from protoplasts come from a single cell, all cells in the transgenic plant should contain the genes of interest that were inserted. It has been shown that foreign genes integrate predominantly at a single locus, are inherited as single mendelian traits, and can be as stable as the wild-type genes (Potrykus et al., 1985a).

The most commonly used procedure for introduction of DNA into protoplasts involves treatment with PEG, much the same as in somatic hybridization. PEG alters the plasma membrane properties by causing a reversible permeability that enable exogenous macro-molecules to enter the

cytoplasm. The exact mechanism of PEG-mediated membrane permeabilization, and thus DNA delivery, is not completely understood (Songstad et al., 1995).

The first report of direct DNA uptake and stable transformation involved the transfer and expression of *Agrobacterium tumefaciens* T-DNA into tobacco protoplasts using PEG (Draper et al., 1982; Krens et al., 1982). The first transgenic plants obtained in this manner were reported by Paszkowski et al. (1984). The keys to being able to accomplish this were optimization of the protoplast to plant regeneration system, development of the aminoglycoside phosphotransferase gene II from *E. coli* transposon Tn5 as a selectable marker conferring plant cell resistance to kanamycin, and optimization of the DNA delivery frequency (Potrykus et al., 1985a).

PEG-mediated transformation is performed in much the same way as protoplast fusion but with the second donor cell being the plasmid as opposed to a second plant protoplast cell. Protoplasts are isolated the same as for fusion, and 3-15 ug of plasmid DNA per ml of protoplasts, is added (Krens et al., 1982; Saul et al., 1988; Paszkowski and Saul, 1988). A variety of different procedures have been used depending on the species being transformed. A number of different parameters have been looked at in order to optimize the systems.

Many early experiments included calf thymus DNA in the protocol (Krens et al., 1982; Krens et al., 1985; Shillito et al., 1985; Peerbolte et al., 1985; Uchimaya et al., 1986), usually at concentrations of 5 times the amount of calf thymus DNA as plasmid DNA. The calf thymus DNA acts as a carrier in the transformation and has been shown to appear in

transformed plants when the transformation frequency is high (Krens et al., 1985; Peerbolte et al., 1985); but has also been reported to not be expressed in those plants (Krens et al., 1985). In many of the early experiments in transformation of protoplasts, calf thymus DNA was reported to be essential for transformation (Krens et al., 1982; Shillito et al., 1985; Negrutiu et al., 1987). However this may not always be the case. Successful transformation has been obtained without using calf thymus DNA or other carrier DNA (Hain et al., 1985; Damm et al., 1989; Tautorus et al., 1989).

Optimization of PEG-mediated transformation is more than likely dependent on a number of factors other than just the presence of carrier DNA. Shillito et al., (1985) demonstrated that, for electroporation (to be discussed later), a PEG concentration of 7.8% w/v was optimum for transformation, particularly when the protoplasts were heat shocked at 45 C for 5 minutes prior to the addition of DNA and PEG. More commonly, particularly for PEG-mediated transformation, an optimum of 20% PEG has been shown (Saul et al., 1988; Maas and Werr, 1989; Armstrong et al., 1990). Damm et al. (1989) showed that with a final PEG concentration of 10-20% the transformation frequency was 3 to 5 times higher than at lower concentrations, but at a PEG concentration of 30 % the frequency was virtually the same. Others have reported success with PEG concentrations of 10% (Tautorus et al., 1989) or 25 % (Antonelli and Stadler, 1989).

Divalent cations are also reported to play a major role in the success of transforming protoplasts. Calcium has been shown to aid in the uptake of DNA by protoplasts. It was thought that calcium phosphate co-precipitates the DNA, which is then taken up by endocytosis (Hain et al.,

1985). This was supported by later research which showed that DNA precipitation is more efficient in the presence of divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}), and efficient DNA incorporation required both PEG and a divalent cation (Maas and Werr, 1989). Ca^{2+} and Mg^{2+} were more efficient than Mn^{2+}. Armstrong et al. (1990) reported higher transformation frequencies with Ca^{2+} than with Mg^{2+}. Other groups have reported higher transformation frequencies with Mg^{2+} than with Ca^{2+} (Negrutiu et al., 1987; Vasil et al., 1988). This difference may be due to additional factors including methods used, the species being transformed, and genotypic effects.

Also reported to play a major role in the efficient transformation of protoplasts is the pH of the PEG solution (Maas and Werr, 1989). This is probably due to the fact that at higher pH (9.0) values large aggregates of DNA are formed that can be taken up less efficiently than the smaller DNA aggregates formed at lower pH (6.0) values.

As mentioned previously, heat shock treatment at 45 C for 5 minutes prior to transformation may increase transformation frequency as in maize (*Zea mays*) (Shillito et al., 1985), sunflower (*Helianthus annuus*) (Moyne et al., 1989), black spruce (*Pinus mariana*), and jack pine (*Pinus banksiana*) (Tautorius et al., 1989). Tyagi et al (1989) found little effect from a heat shock treatment on tobacco (*Nicotiana tabacum*).

Electroporation is often used either alone or in combination with PEG. Electroporation involves the subjecting of protoplasts to electrical pulses of AC and DC current to cause reversible permeabilization of the plasma membrane enabling macromolecule delivery. Isolated protoplasts and the plasmid are mixed prior to electroporation.

An AC field giving 25 volts (peak-to-peak) at 500 KHz which aligns the protoplasts in chains of varying lengths is applied. Excessively long chains are avoided by using a voltage strong enough to bring about alignment, but applied at a slow rate of increase over a five minute period. A DC pulse of 2 milliseconds is given and a few seconds later the AC field is progressively reduced to zero. (Power et al., 1988). Many of the earlier successes in stably transforming tobacco protoplasts involved combinations of PEG and electroporation treatments (Shillito, 1985; Fromm et al., 1986; Negrutiu et al., 1987; Saul et al., 1988). Electroporation is commonly used for transient gene expression studies in both mammalian and plant protoplast systems (Songstad et al., 1995).

Other Methods of Transformation

Microinjection.

Microinjection is performed under the microscope with the DNA injected directly into individual cells. Microinjection has the advantage in that the amount of DNA injected into a cell is not limited, the delivery is precise, and cells with high regeneration capacity can be targeted. Microinjection is, however, very labor intensive and requires a highly skilled individual to perform the injection (Potrykus, 1990). In haploid canola, microspore-derived embryoids microinjected at the 12 cell stage of development resulted in the recovery of transformed plants, but they were chimeric and required recovery by secondary embryogenesis (Neuhaus et al., 1987). Protoplasts have been shown to survive microinjection (Griesbach, 1985; Lawrence and Davies, 1985; Morikawa and

Yamada, 1985 a,b; Reich et al., 1986a,b; Steinbiss and Stabel, 1983), as have pollen grains (Crossway et al., 1986; Kranz and Lorz, 1990) and onion epidermal cells (Bradley, 1979). However, transformation by microinjection of protoplasts has not been reported (Songstad et al., 1995).

Liposome Mediated Transformation.

Liposomes entrap a large variety of macromolecules such as enzymes or nucleic acids and can be used to deliver them into cells by fusion with the plasma membrane or by endocytosis. The choice of the proper lipid composition is very important to the system. Most of the successes using liposomes has been with animals (Gad et al., 1990). The mode of action of DNA delivery is unclear, but there is evidence that it is by cellular uptake (endocytosis) as determined by electron microscopy (Fukunga et al., 1983; Nagata, 1984; Guerineau and Tailliez, 1986). Negatively charged liposomes work better than positively charged liposomes (Gad et al., 1986). The genetic material encapsulated in negatively charged liposomes is inserted into the cells usually by the addition of fusogens to the reaction mixture. Divalent cations such as Ca^{2+} promote liposome fusion (Gad et al., 1988).

Protoplasts appear to be best suited for receiving exogenous DNA from liposomes. Both transient and stable gene expression has been obtained in tobacco protoplasts using liposome-encapsulated DNA (Rosenberg et al., 1988). The traits transformed to the zygotic offspring were shown to be inherited as one dominant gene (Deshayes et al., 1985).

Transformation Using Silicon Carbide Fibers.

DNA delivery into plant cells using silicon carbide fibers was first reported by Kaepler et al. (1990). This simple method involves vortexing a mixture of plant cells, plasmid DNA and silicon carbide fibers. Silicon carbide fibers likely mediate DNA delivery because of their shape, size, strength, and chemical composition. However, silicon carbide fibers pose a lung disease and carcinogenic danger similar to asbestos, if inhaled (Songstad et al., 1995).

Cross Protection

Cross protection can be defined as the use of a mild or attenuated strain of a virus to prevent further infection from more severe strains of a virus or to other closely related viruses (Sequiera, 1984; Fulton, 1986; Palukaitis and Zaitlin, 1984; Wilson, 1993). A variety of terms have been applied to this phenomenon including acquired immunity, antagonism, cross immunization, dominance, interference, premunity, induced immunity, prophylactic inoculation and protective inoculation (Fulton, 1986).

The first report of cross protection was made in 1929 when McKinney, working with tobacco mosaic virus, reported the ability of one strain of the virus to protect against later infections by a second strain of the virus (McKinney, 1929). Later Salaman (1933) found that tobacco plants infected by a mild strain of potato virus X were protected against other strains of this virus. Salaman proposed that this cross protection could have economic importance, and since then there have been numerous attempts to use avirulent strains to improve the yield of many crops, but with

limited success (Fletcher, 1978). However, for a number of viruses this approach has been successfully applied. These include tomato mosaic virus (Broadbent, 1976), papaya ringspot virus (Yeh et al., 1988), tobacco mosaic virus (Rast, 1972), and citrus tristeza virus (Muller and Costa, 1966, 1969, 1977; Costa and Muller, 1980).

The mechanisms by which cross protection works are not well understood (Gadani et al., 1990). Earlier theories on the mechanism of action of cross protection centered around four general categories. First was the theory of precursor exhaustion in which the inducing virus has exhausted one or more of the essential materials required for the replication of the challenging virus. The second theory is that the inducing virus produces substances which specifically inhibit related virus, but not unrelated viruses. It would be possible for this specific inhibitor to bind to the incoming challenging virus. A possibility for such a specific inhibitor would be the viral replicase. A third theory is that the inducing virus has altered the metabolism of the host to such an extent as to render it insusceptible to a challenging virus. Finally, a fourth theory centers around coat protein sequestration. The coat protein of the inducing virus encapsidates the nucleic acid of the related challenging virus, and the virions that are then produced are not in an environment of the cell enabling their nucleic acid to be released and replication to begin. (Sherwood and Fulton, 1982; Palukaitis and Zaitlin, 1984). However, none of these theories can be shown to be correct for all viruses, and in fact the true mechanism may be a combination of these or an as yet undescribed mechanism.

Cross protection of CTV in Citrus

In order to have effective cross protection the mild isolate used must be mild in all citrus cultivars and susceptible hosts grown in a given area. In addition, the virus used must be genetically stable with little or no chance of reverting to a severe form. Artificially attenuated or mutated viruses might be more likely to revert back to the severe form. Finally, the slight reduction in yield and performance of the host due to the effects of the mild strain should be economically acceptable (Lee and Rocha-Pena, 1992).

The first report that cross protection could be possible in citrus was made in 1951 (Grant and Costa, 1951). Most early work on cross protection of citrus was done to protect against the stem pitting isolates of CTV. Consequently, a great deal of work has been done in countries where severe stem pitting isolates exist. By 1980 over 15 million 'Pera' sweet orange trees were inoculated for protection against CTV-induced stem pitting in Brazil (Costa and Muller, 1980). Mild strain cross protection has been used on grapefruit in Japan (Sasaki, 1979; Leki, 1989; Koizumi et al, 1991), Australia (Stubbs, 1964; Cox et al., 1976; Thornton et al., 1980), South Africa (De Lange et al., 1981) and Venezuela (Romero et al., 1991). Also reported was cross protection of naval orange in Japan (Koizumi, 1991), and limes in India (Balaraman and Ramakrishnan, 1980) and Venezuela (Romero, et al., 1991).

Because most countries abandon sour orange as a rootstock when CTV-induced quick decline occurs, relatively little research has been done on cross protection against quick decline. However, because of its excellent

horticultural characteristics, there has been recent interest in evaluating whether mild strain cross protection would work to prevent quick decline of trees on sour orange rootstock. Thornton and Stubbs (1976) reported a delay in the onset of tristeza induced quick decline of 'Marsh' grapefruit on sour orange. However, by 1980 they were reporting some breakdown in the immunity of these trees (Thornton et al., 1980). Wallace and Drake (1976) also reported a delay in symptoms of 'Valencia' sweet orange on sour orange after preimmunization. Van Vuuren et al. (1991) evaluated 11 mild CTV isolates from Florida, Israel, and South Africa for cross protection of 'Valencia' sweet orange on sour orange in South Africa. Quick decline symptoms appeared after 4 years for some of the isolates. Only one isolate appeared to have potential commercial value under the South African climatic conditions. In a similar study in Florida, 'Hamlin' sweet orange and 'Redblush' grapefruit trees on sour orange rootstock were preinfected with 14 different mild strain isolates of CTV. These trees were exposed to natural challenge in an area where severe decline strains were prevalent. The CTV decline was more prevalent in the 'Hamlin' trees than in the 'Redblush' Grapefruit (Yokomi, et al., 1991). Another field trial by Rocha-Pena et al. (1991) showed little or no decline after four years for 'Hamlin' and Grapefruit on sour Orange, suggesting that with proper selection of mild CTV isolates, cross protection against quick decline on sour orange is possible.

There are, however, several problems involved in the use of cross protection as a control strategy against severe strains of citrus tristeza virus (Lee and Rocha-Pena, 1992). First, the budwood source trees have to be biologically indexed on a frequent basis to ensure that a severe strain

has not been introduced (De Graca et al., 1982). The erratic distribution of CTV within the scion can result in as much as 50% of the buds being propagated that should contain the mild strain isolate, actually being virus free (Lee et al., 1987). Hot weather can induce a kind of thermotherapy which often prevents the spread of the mild strain isolates, thus leaving parts of the tree virus free and exposed to infection from severe isolates, breaking down the cross protection (Lee et al., 1988). Selection of the mild isolates to use can be very difficult and inefficient (Costa and Muller, 1980). This may be partly due to the fact that many isolates which show cross protection in one environment may express severe symptoms or at least fail to cross protect in other environments (Lee and Rocha-Pena, 1992). It is these problems which have contributed to the idea of genetically engineering the cross protection into citrus.

Genetic Engineering for Virus Resistance

With the dramatic advances in plant transformation technology there has been a tremendous interest in introducing virus resistance by genetic engineering. With a few exceptions, the genes used to induce this virus resistance have come from the genome of the virus. This is partly due to the difficulties still inherent in isolating plant resistance genes from the relatively large plant genome. In contrast, plant viruses contain relatively few genes which can be more readily identified and cloned. A majority of the examples of genetically engineered viral resistance have

involved the use of the coat protein gene but other genes have been used to obtain virus resistance as well.

Replicase Gene

The first report of replicase mediated resistance was demonstrated with tobacco mosaic virus (Golemboski et al, 1990; Carr et al., 1992). While attempting to determine the role of a putative 54-kDa protein encoded within the replicase of TMV, they found that the plants expressing the 54-kDa protein were completely resistant to TMV, even at inoculum levels up to 1000 fold higher than conferred by the TMV coat protein gene. Donson et al., (1993) demonstrated no resistance to TMV in tobacco plants transformed with the full length (183 kDa) replicase gene, but in transgenic plants with an additional insertion which would terminate translation in the middle of the 183 kDa gene, a high level of resistance to systemic infection by TMV and other tobamoviruses was noted. Similar results were obtained with cucumber mosaic virus (CMV) in tobacco (Anderson et al., 1992), potato virus X (PVX) in tobacco (Braun and Hemenway, 1992; Longstaff et al., 1993) pea early browning virus (PEBV) in *Nicotiana benthamiana* (MacFarlane and Davies, 1993), potato virus Y (PVY) in tobacco (Audy et al., 1994), and cymbidium ringspot virus (CyRSV) in *N. benthamiana* (Rubino et al., 1993). Rubino and Russo (1995) later reported that the transgenic *N. benthamiana* plants containing the CyRSV full-length replicase gene were not resistant to the closely related artichoke mottled crinkle tombusvirus (AMCV) and carnation italian ringspot tombus virus (CIRV), indicating that the resistance was probably RNA-mediated resistance. In most of these cases the resistance of the most resistant

replicase expressing lines was greater than for the coat protein expressing lines. No field testing of the replicase expressing transgenic plants has yet been reported.

Movement Protein

Tobacco plants expressing a defective movement protein of TMV or transformed with the movement protein from brome mosaic virus (BrMV), which cannot spread in tobacco, were shown to be protected from TMV (Malyshenko et al., 1993). The authors theorized that the presence of either of these movement proteins before viral inoculation inhibited the ability of the incoming functional viral movement protein from TMV to successfully interact with the plasmadesmata.

Protease

The potyvirus genome codes for a single large polypeptide that is subsequently cleaved into individual mature viral proteins through the action of three viral encoded proteases (Dougherty and Carrington, 1988). One of these genes which encodes the NIa protein was used to transform tobacco (Vardi et al., 1993). Of the 50 transgenic plants produced, 2 were found to be resistant to PVY. However the authors believe that this resistance may be due to accidental alterations which may have occurred during the cloning of the gene which may have resulted in a dysfunctional protein. Nevertheless, this may lead to a possibility of intentionally modifying the protease genes of viruses which produce polyproteins to obtain virus resistance.

Antisense and Sense-defective RNAs

There have been several reports using antisense or sense-defective RNAs to engineer virus resistance. It is thought that the interaction between complementary transgene and viral encoded gene sequences interferes with translation, replication or viral nucleic acid stability (Grumet, 1995). Cuozzo et al. (1988) transformed tobacco plants with the cauliflower mosaic virus (CMV) antisense coat protein (CP). They found resistance only at low inoculum levels indicating that antisense CP-RNA resistance was less effective than the coat protein mediated resistance. Similar results were shown for PVX (Hemenway et al., 1988), TMV (Powell-Abel et al., 1989), and zucchini yellow mosaic virus (ZyMV) (Fang and Grumet, 1993). The lack of protection at higher inoculum levels may be due to a gene dosage effect (Gadani et al., 1990). The mechanism of action of viral antisense RNA may be due to an inhibition of coat protein synthesis by the formation of an antisense-sense RNA hybrid, prevention of replication by binding of the antisense RNA to the origin of replication or by competition with the viral negative strand for viral or host components necessary for replication (Gadani et al., 1990).

There are, however, examples where RNA mediated protection was much more effective, most notably in the luteovirus and potyvirus groups. Lindbo and Dougherty (1992a,b) demonstrated that both sense and antisense forms of the coat protein gene of Tobacco Etch Virus (TEV) conferred a high level of resistance to TEV. Hammond and Kamo (1993) demonstrated varying degrees of resisance in their *N. benthamiana* plants transformed with the antisense coat protein of bean yellow mosaic potyvirus (BYMV). Kawchuk et al., (1991) showed that both the sense and antisense RNA of the

coat protein gene of potato leaf roll luteovirus (PLRV) showed a high level of resistance to PLRV infection.

Coat Protein

Since the phenomenon of cross protection was first observed over 65 years ago by McKinney (1929) there has been considerable interest in the development of plants protected from severe virus infection. There have been several studies which have indicated that the coat protein plays a major role in cross protection (DeZoeten and Fulton, 1975; Sherwood and Fulton, 1982; DeZoeten and Gaard, 1984; Wilson and Watkins, 1986; Zinnen and Fulton, 1986). In order to test this hypothesis, Powel-Abel and her colleagues introduced the capsid protein gene of tobacco mosaic virus into tobacco plants by using a chimeric gene construct containing a cDNA from the coat protein coding sequence, with the CaMV 35S promoter and the polyadenylation seguence from the *Agrobacterium* nopaline synthase gene. After *Agrobacterium*-mediated transformation and subsequent plant regeneration, these plants were tested for virus resistance. They determined that high levels of accumulation of coat protein corresponded to high levels of resistance to virus infection, and a corresponding delay in symptom development in the progeny of self-fertilized transgenic plants. The protection was overcome by inoculation of the transgenic seedlings with unencapsidated viral RNA and was less effective when a high virus concentration was used (Powel-Abel et al., 1986) This mimics what happens with classical cross protection with TMV. Since this first successful demonstration of coat protein-mediated resistance (CP-mediated

resistance), there has been a considerable interest in using this approach in a variety of plant species (See Table 2-2).

There is no consensus on how CP-mediated resistance works. In fact, the mechanism may be dependent on the virus-plant-coat protein system and the exact nature of how the protection works may depend on the viral gene construct used, transgene position effects, copy number, transcriptional activity, the extent and site of uninhibited virus replication and spread within the host plant, and secondary effects of transgenesis on host cell metabolism and general stress anddisease resistance responses (Wilson, 1993). For example, in the case of TMV (Powel-Abel et al., 1986), alfalfa mosaic virus (AlMV)(Loesch-Fries et al., 1987), and potato virus X (PVX)(Hemenway, 1988) the level of resistance correlated positively with the levels of coat protein in the transgenic plants. Plants that only accumulated the coat protein transcript of TMV or AlMV and not the coat protein itself were not resistant. However, in plants transformed with the coat protein gene of potato virus Y (PVY) (Farinelli and Malnoe, 1993) and potato leaf roll virus (PLRV) (Barker et al., 1993), resistance correlated with the levels of coat protein transcripts and not with the levels of coat protein. Moreover, in the case of PVY and PLRV some virus resistant transgenic lines were identified in which the coat protein was not detectable. To further complicate things, tobacco transformed with the coat protein gene of tomato spotted wilt virus (TSWV) were resistant when an intact coat protein gene was used, as well as when the transgene was rendered untranslateable through the removal of the initiating start codon (DeHaan et al., 1992). This would indicate that expression of the coat protein is not necessarily required for resistance.

Table 2-2. Examples of coat protein-mediated resistance.

Virus	Plant	Resistance	Reference
Tobacco Mosaic Virus	tobacco	delay	Powel-Abel et al., 1986
	tobacco	to other Tobamo-also	Nejidat and Beachy, 1990
	tomato		Nelson et al., 1988
Alfalfa Mosaic Virus	alfalfa	yes	Halk et al., 1989
	tobacco	delay	Loesch-Fries et al., 1987
	tomato	yes (tobacco also)	Tumer et al., 1987
Tobacco Rattle Virus	tobacco	yes- Pea Early Browning Virus also	van Dun and Bol, 1988
Tobacco Streak Virus	tobacco	yes?	van Dun et al., 1987
Cucumber Mosaic Virus	cucumber	yes	Gonsalves et al., 1991a
	cucumber	field tested resistance	Gonsalves et al., 1992
	tobacco	yes	Cuozzo et al., 1988
	muskmelon	yes	Gonsalves et al., 1991b; Gonsalves et al., 1994
Tomato Spotted Wilt Virus	tobacco	yes	MacKenzie and Ellis, 1992
	tomato	yes	Ultzen et al., 1995
	chrysanthemum	not tested	Yepes et al., 1995
Potato Virus Y	potato		Lawson et al., 1990
Lettuce Mosaic Virus	tobacco	yes	Dinant et al., 1993
Papaya Ringspot Virus	papaya	yes	Fitch et al., 1992
	plum		Scorza et al., 1991

Virus	Plant	Resistance	Reference
Bean Yellow Mosaic Virus	tobacco		Hammond and Kamo, 1991
Plum Pox Virus	plum	yes	Scorza et al., 1994
	tobacco	variable	Ravelenandro et al., 1993
Tobacco Vein Mottling Virus	tobacco		Main et al., 1991
Soybean Mosaic Virus	tobacco	broad spectrum	Stark and Beachy, 1989
Potato Leaf Roll Virus	tobacco	yes	Kawchuck et al., 1990
	potato	+/- sense	Kawchuck et al., 1991
Potato Virus X	tobacco	+/- sense	Hemenway et al., 1988
	potato	yes	Hoekema et al., 1989
	potato	yes- field tested	Jongdijk et al., 1992
Cassava Mosaic Virus	tobacco		Fauquet et al., 1991
Potato Virus S	tobacco		Mackenzie and Tremaine, 1990
Rice Stripe Virus	rice	yes	Hayakawa et al., 1993
Grapevine Fan Leaf Virus	grape	yes	Bardonnet et al., 1994
	grape	variable	Krastanova et al., 1995
Maize Dwarf Mosaic Virus	maize	yes- also MCMV*	Murry et al., 1993
Tomato Yellow Leaf Curl Virus	tomato	yes	Kunik et al., 1994
Arabis Mosaic Virus	tobacco	yes	Bertioli et al., 1992

* - MCMV is Maize Chlorotic Mottle Virus

Table 2-2 (continued). Examples of coat protein-mediated resistance.

In the potyviruses (PVY, TEV, ZYMV), and luteoviruses (PLRV), when intentionally truncated antisense or nonexpressing (with no start codon) coat protein genes were transformed into plants, there was a measurable or even complete protection against the parent virus (Kawchuck et al., 1991; van der Wilk et al., 1991; Lindbo and Dougherty, 1992a,b; DeHaan et al., 1992; van der Vlugt et al., 1992; Fang and Grumet, 1993; Farinelli and Malnoe, 1993; Lindbo et al., 1993). In all these cases the resistance may be due to some direct form of RNA-RNA interference between the transgene transcript and challenge virus. In TEV with a truncated CP gene, it was common for the inoculated leaves to develop symptoms and virus titer which was similar to that of control plants, but for the plants to outgrow the infection, suggesting some interference with virus spread (Lindbo and Dougherty, 1992a).

Recently, Dougherty and his colleagues have studied in detail coat protein-mediated resistance using TEV. Their results confirm that transgene-derived RNA may be either translatable or untranslatable indicating that a product of the transcript is not involved in eliciting the resistance. That is, plants displaying the virus resistance phenotype transcribe the transgene at a high rate, yet accumulate the transcript at a low level (Dougherty et al., 1994; Smith et al., 1994; Mueller et al., 1995). In plants which exhibit these characteristics, the cytoplasmically located virus does not replicate to a detectable level. It has been suggested that a post-transcriptional RNA targeting system could be activated, resulting in the elimination of both transgene and viral RNA from the cytoplasm (Lindbo et al, 1993; Dougherty and Parks, 1995).

Dougherty and his colleagues (Smith et al., 1994) have further suggested that there is a threshold level of transgene-derived transcripts that must be exceded in order to activate the cytoplasmic post-transcriptional RNA degradation process and thus elicit virus resistance. This suggests that the number of transgenes and their level of expression are important in determining the activation state of the cytoplasmic system. To further explore this RNA-mediated virus resistance Dougherty and his colleagues (Goodwin et al., 1996) undertook a study to characterize the biochemical and genetic processes of the resistance. Using a set of plants that contained zero, one, two, or three copies of the transgene in both a homozygous and heterozygous condition, it was determined that three or more transgenes were necessary to establish the highly resistant state. One or two transgene copies resulted in an inducible form of resistance. The steady state RNA levels and the rates of transcription of the transgenes in the plants in this study supported a post-transcriptional RNA degradation process as the underlying mechanism for transgene transcript reduction and virus resistance. Resistance appears to be initiated by a cleavage of specific sites within the target RNA sequence. Further experiments are being conducted to see if this really is the case.

Transformation of Citrus

The first successful transformation of Citrus was reported by Vardi et al. (1990) using PEG-mediated direct DNA uptake. They were able to successfully transform Rough Lemon (*Citrus jambhiri*) protoplasts. The first successful

transformation of Citrus by *Agrobacterium tumefaciens* was reported by Moore et al. (1992). Transgenic Carrizo Citrange (*C. sinensis* X *Poncirus trifoliata*) were produced by co-culturing with *Agrobacterium tumefaciens*. Transformation and stable integration in regenerated plants was confirmed by PCR and Southern analyses. Hidaka et al. (1990) reported the recovery of transgenic 'Washington Navel' sweet orange (*C. sinensis*) from *Agrobacterium*-mediated transformation. Kobayashi and Uchimaya (1989) reported the transfer of foreign DNA into sweet orange protoplasts by direct DNA uptake but since no plants were regenerated, stable transformation can not be claimed. Schell (1991) reported the transformation of 'Hamlin' sweet orange protoplasts and the regeneration of transgenic plants as demonstrated by histochemical assaying for GUS and PCR analysis, but not by genomic Southern analysis. Pena et al. (1995) reported the transformation and subsequent regeneration of transgenic plants from sweet orange by *Agrobacterium* mediated transformation. Integration of the UidA (GUS) gene was confirmed by Southern analysis.

Attempts are now being made by several labs to insert the coat protein gene from citrus tristeza closterovirus (CTV) into citrus. It is hoped that this genetically engineered cross protection will result in the production of trees resistant to the tristeza virus, which is one of the most economical damaging pests of *Citrus*. Espinosa (1995) successfully introduced the coat protein gene of CTV into 'Carrizo' citrange (*C. sinensis* X *Poncirus trifoliata*) and sour orange. These results have been confirmed by Southern and western immunoblot analyses. These are the only results so far confirmed.

In addition, Febres-Rodriguez (1995) has characterized the genes for three other open reading frames (P18, P20, P27) and has attempted to transform Citrus with these genes. He showed the production of plants which were GUS positive and positive by PCR but not confirmed by Southern analysis.

CHAPTER 3

MATERIALS AND METHODS

Plasmid Preparation

The two plasmids used in this research, were supplied by the laboratory of Dr. C.L. Niblett. The two plasmids used were pMON10098 (provided by Dr David Stark, Monsanto Corp. to Dr. Niblett) and pBPFΩ-7 (provided by Dr. G. Selman, Institute for Genetic Engineering (Havana, Cuba, to Dr. Niblett). The coat protein genes from Florida isolate T30 (a mild isolate) of CTV, and Florida isolate T36 (a severe decline isolate) of CTV were inserted into each of the two plasmids. The only difference in the transformations into *Escherichia coli* for the two plasmids was that pMON10098 was selected for using spectinomycin (75μl/ml), and pBPFΩ-7 was selected for using ampicillin (50μg/ml). The plasmid pMON10098 contains an enhanced CaMV 35 S promoter, nos terminator, and the NPTII gene. The plasmid pBPFΩ-7 contains two CaMV 35 S promoters in tandem, plus the 71 base pair non-coding 5' leader sequence of TMV (TMV Ω) attached to the 3' end of the nos terminator, in pUC18 (See Figure 3-1). A detailed map of the pUC18 plasmid can be found in Sambrook, et al. (1989). Neither plasmid contained a scoreable marker such as the uidA gene for β-glucuronidase.

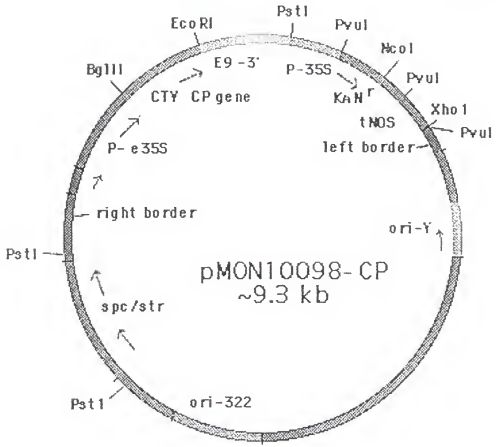


Figure 3-1a. Map of pMON10098. Spc/str is the Spectinomycin and streptomycin resistance gene. P-e35S is the enhanced CaMV 35S promoter. CP is the coat protein gene. E9 3' is the terminator sequence. P-35S is the CaMV 35S promoter. Kan is the Kanamycin resistance gene (equivalent to the NPTII gene). Nos-3' is the Nopaline synthase terminator. ori-V is the origin of replication. R is the right border and L is the left border. The -> or <- is the direction of translation. The Kan gene confers resistance in the plant cells. The spc/str gene allows for selection in *E. coli*. The rest of the plasmid that is not shown is pBR322 (See Sambrook et al., 1989 for the map of pBR322).

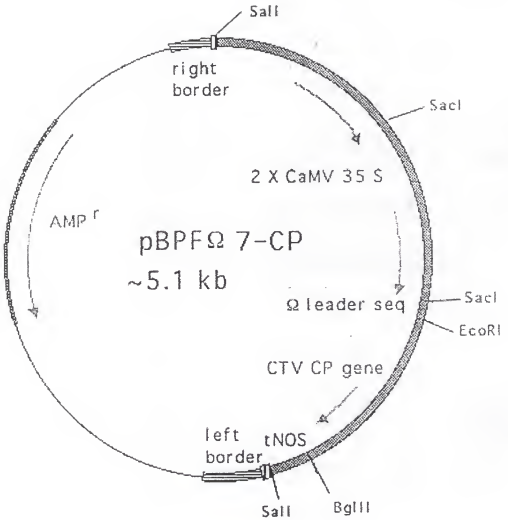


Figure 3-1b. Map of pBPFΩ-7. amp is the ampicillin gene used for bacterial selection. P-35S is the enhanced CaMV 35S promoter which consists of two CaMV 35S promoters in tandem. CP is the coat protein gene. Ω is the 71 base pair 5' leader sequence of TMV. The rest of the plasmid is pUC18 (See Sambrook et al., 1989 for the map of pUC18).

Transformation of E. coli (Sambrook et al., 1989; E. Anderson, pers. comm.).

Before a large scale plasmid preparation can be performed, the plasmid must first be transformed into competent E. coli cells. Competent cells were prepared using the following procedure (E. Anderson, pers. comm.). An microfuge tube containing 1.5 ml of 2xYT medium (16 g/l bactotryptone, 10 g/l yeast extract, 5 g/l NaCl) was inoculated with E. coli strain HB101 and incubated overnight on a shaker at 225 RPM at 37°C. To 50 ml of 2xYT in a 125 ml Erlenmeyer flask was added 125 µl of the bacterial culture. The bacterial culture was then incubated at 37°C at on a shaker at 225 RPM. After 2 hours 10 ml of this bacterial culture was removed and placed in a prechilled Falcon 2056 tube and left on ice for 15 minutes. The sample was then centrifuged for 10 minutes at 5000 RPM in a J-20 rotor. The supernatant was decanted off and the pellet resuspended in 5 ml of 50 mM $CaCl_2$ (sterile), vortexed, and incubated on ice for 15 minutes. The sample was again centrifuged at 5000 RPM for 10 minutes in a J-20 rotor and the supernatant decanted off. The pellet was then resuspended in 500 µl of 50 mM $CaCl_2$ by gently tapping the sides of the tube and placed back on ice for at least two hours. The cells were then ready to be used or could be stored at -80°C (with 50% glycerol added) until needed for transformation with the plasmid.

To a prechilled Falcon 2056 tube was added 200 µl of competent cells along with 1-2 µl of plasmid. The cells and plasmid were gently mixed by tapping the sides of the tube and the sample left on ice for 30 minutes. The sample was then heat shocked for 2 minutes at 42°C and immediately

placed back on ice for 5 minutes. To this sample was then added 1 ml of 2xYT and the sample gently mixed by tapping the sides of the tube, and the samples incubated for 1 hour at 37°C shaking at 225 RPM. After one hour 100 µl of sample was plated onto LB solid media (10 g/l bactotryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g.l agar) containing the appropriate antibiotic, and incubated at 37°C inverted in a 37°C incubator.

The surviving colonies were selected for further use by transferring cells from the colony with a sterile toothpick which was then swirled in an microfuge tube containing 1 ml of 2XYT supplemented with 100 µg/ml of either spectinomycin or ampicillin (depending on the plasmid). These cells were then grown overnight and tested for presence of the plasmid by doing a plasmid miniprep, and then stored at -20 for future use.

Quick Miniprep Plasmid Purification. (from E.J. Anderson, pers. comm.)

Plasmid DNA was extracted from bacterial increases of several of the colonies and compared to the DNA from the known plasmid stock by restriction analysis using agarose gel electrophoresis in the following manner.

Single colonies were selected from the plated cultures using a sterile toothpick to transfer cells from the individual colonies to an microfuge tube containing 1.5 ml of 2xYT media and the appropriate antibiotic, and grown overnight at 37 C, shaken at 225 RPM. The samples were pelleted by centifuging in a microfuge for 2 minutes. The supernatant was discarded and the tube shaken vigorously to remove as much of the media as possible, and set upside down on a kimwipe to dry briefly.

The cells were then resuspended by gentle vortexing after adding 250 µl of SucTET (8.8 g Sucrose, 1 ml of 1M Tris-HCl pH 8.0, 10 ml of 0.5 M EDTA, 500 µl Triton X100, volume adjusted to 100 ml) plus 15 µl of lysozyme (from 10 mg/ml stock), and placed on ice for 10 minutes. The cells were then boiled for 50 seconds and placed on ice for another 10 minutes. The cellular debris was pelleted by centrifuging for 10 minutes in a microfuge. The debris was then removed using a sterile toothpick. To the remaining liquid was added 65 µl of 7.5M ammonium acetate, and the samples incubated for 10 minutes on ice. The samples were centrifuged for 5 minutes and the supernatant transferred to a fresh tube. To each sample was added 200 µl of isopropanol, the sample mixed by inversion, kept on ice for 20 minutes, and then centrifuged for 10 minutes. The supernatant was poured off, the pellet gently washed with 70% ethanol, and dryed under vacuum. The pellet of plasmid DNA was then reuspended in 50 µl of TE buffer.

A restriction digest was then performed to test for the presence of the coat protein gene. First, 5 µl of the isolated plasmid (plus a control of the original plasmid) was removed and placed in an microfuge tube. To this was added 5 µl of 10X of the restriction enzyme buffer H (Promega), 1.0 µl of EcoRI (Promega), and 39µl of distilled water, and the restriction digest allowed to proceed by incubating for at least 60 minutes at 37°C. The plasmid was then ethanol precipitated by adding 5 µl 3 M sodium acetate and 200 µl of 95% ethanol (cold) and centrifuging for 15 minutes. The supernatant was discarded and the pellet washed by carefully adding 250 µl of 70% ethanol (cold) and centrifuging for another 5 minutes. The supernatant was then discarded and the pellet dried under

vacuum. The pellet was then resuspended in 44 µl of sterile ddH_2O and 5 µl of restriction enzyme buffer D (Promega) and 1.0 µl of BglII was added. The samples were placed at 37°C for 1 hour and ethanol precipitated as before. The dried sample was then resuspended in 10 µl of TE buffer. Meanwhile, an agarose gel (1% agarose in TBE (see Appendix B)) was prepared. Ten minutes before loading the gel 0.5 ml of RNase (10mg/ml) was added to each tube. Indicator dye (3 µl of 6X loading buffer) was added and the gel was loaded. The gel was run for 1 to 2 hours at 100 Volts. The gel was then removed and stained with 10 µl of ethidium bromide (10 mg/ml) in 100 ml of distilled water with gentle shaking for 30 minutes or longer. The gel was de-stained in distilled water (approx. 100 ml) for at least 5 to 10 minutes, then observed and photographed on a UV light box. Samples which contained the band corresponding to the band for the coat protein gene (as observed in the control) were saved to use for the large scale isolations.

Large Scale Isolation and Preparation of Plasmid (Sambrook et al., 1989)

The day before plasmid isolation, four 1-liter flasks containing 500 ml of Luria Broth plus the appropriate antibiotic were inoculated with the transformed E. coli and the cells were incubated overnight at 37°C on a shaker at 225 rpm.

The next morning the bacterial cultures were dispensed into 500 ml centrifuge bottles and centrifuged at 5000 rpm in a JA-10 rotor. The supernatant was discarded and the bacterial pellet resuspended in 20 ml of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, with 5 mg/ml lysozyme added just before use). The solution was allowed to

stand at room temperature for 5 minutes and then 40 ml of freshly prepared Solution II (0.2 M NaOH, 1% SDS) was added. The solution was then gently mixed by inverting the bottles gently several times and allowed to set at room temperature for 10 minutes.

The contents of each bottle were then split between two 50 ml centrifuge tubes. To each tube was added 10 ml of ice-cold Solution III (60% 5 M potassium acetate, 11.5% glacial acetic acid, 28.5% distilled H_2O). The tubes were covered with a cap, mixed by shaking the tubes several times, and placed on ice for 10 minutes. At this time a flocculent white precipitate formed. The samples were then centrifuged at 10,000 rpm (JA-20 rotor) for 10 minutes at 4°C and the rotor allowed to stop without braking.

The supernatant from all eight tubes was filtered through four layers of cheesecloth and into a 500 ml centrifuge bottle. The solution was then equally divided between two 500 ml centrifuge bottles. To each bottle, 100 ml of isopropanol was added and the bottles were placed at room temperature for 30 minutes or longer.

The nucleic acids were recovered by centrifuging at 5000 rpm (JA-10 rotor) for 30 minutes at 4°C. The supernatant was decanted off carefully and discarded. The pellet was washed with 200 ml of cold 70% ethanol and centrifuged again for 10 minutes at 5000 rpm at 4°C. The alcohol was poured off carefully and the pellet allowed to air dry. After air drying the pellet was resuspended in 10 ml of TE buffer (see Appendix B) and allowed to set at room temperature for at least an hour to allow for as much of the plasmid as possible to go into solution. The solution was transferred to a 50 ml centrifuge tube and 5 ml of chloroform-isoamyl

alcohol (24:1) and 5 ml of tris-equilibrated phenol (Sambrook et al. 1992) was added. After thoroughly mixing, the solution was centrifuged at 5000 rpm (JA-20 rotor) for 10 minutes to separate the phases. The aqueous (upper) phase containing the plasmid was removed and placed in another 50 ml centrifuge tube. The plasmid was ethanol precipitated by adding 3.5 ml of sodium acetate and 35 ml of ethanol and placed in the refrigerator at 4°C overnight, followed by centrifuging for 30 minutes at 10,000 rpm (JA-20 rotor) at 4°C.

Immediately after centrifuging the supernatant was decanted, 10 ml of cold 70% ethanol added, and the tubes were recentrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant was immediately decanted and the pellet dried under vacuum. The pellet was then resuspended in 10 ml of TE buffer. To determine the concentration of plasmid DNA, an absorbance reading at an $O.D._{260}$ was obtained. An absorbance reading of 1.0 corresponds to 50 μg of plasmid DNA per ml. The plasmid was ethanol precipitated as before and resuspended at 1 $\mu g/\mu l$ in BH3 (See Appendix A).

Establishment and Maintainance of Suspension Cultures

The *Citrus* suspension cultures used for protoplast isolation were obtained from ovule-derived callus of 'Nova' tangelo ('Clementine' mandarin (*Citrus reticulata* Blanco) X 'Orlando' tangelo (*C. reticulata* X *C. paradisi* Macf)) or 'Rohde Red Valencia' sweet orange (*C. sinensis* L(Osbeck)). The suspensions were maintained in 40-50 ml of liquid 1/2-1/2 medium (see Appendix A) in 125 ml erlenmeyer flasks covered with aluminum foil and sealed with masking tape. Cultures were maintained on a shaker at 140 rpm with no supplemental lighting. These suspensions were

subcultured every two weeks by splitting the contents between two sterile flasks and adding a half volume (25 ml) of fresh culture medium to each flask. The cells were used for protoplast isolation 5-14 days after subculture.

Isolation and Transformation of Citrus Protoplasts

Isolation of protoplasts from suspension cultures was performed as follows (Grosser and Gmitter, 1990, as modified by Schell (1991)). First, one ml of suspension culture was placed in a 10 mm X 60 mm petri plate (usually 2 to 3 plates for each suspension used). Three ml of 0.7 M BH3 was added followed by 1 ml of enzyme solution (see Appendix A). Plates were wrapped with Nesco film and placed on a shaker at 50 rpm inside an incubator at 28°C overnight (16 hours).

Protoplasts were collected by passing the above mixture through a sterile 0.45 micron stainless steel screen (which had been adhered to the top section of a 30 cc disposable syringe) and into a 50 ml collection tube. This mixture was then transferred into a sterile 15 ml glass conical centrifuge tube with a screw cap. The cells were then pelleted by centrifuging for 5 minutes at 500 rpm (75 x g).

The supernatant was removed using a sterile pasteur pipet and the pellet was resuspended in 4 pipets (approx. 6-7 ml) of CPW25SUC (see Appendix A). Then the solution was overlayed with 5 pipets (approx 7-8 ml) of CPW13MAN (See Appendix A). The CPW13MAN was added slowly, and the tube was slanted so that the two solutions were not mixed. At this point there were two distinct layers visible- a lower layer containing the

protoplasts and debris, and an upper clear layer. They were then centrifuged for 10 minutes at 500 rpm (75 x g).

After centrifugation a distinct band was visible at the interphase of the two solutions, a pellet at the bottom of the tube, and a cloudy appearance between the two. The band at the interphase layer contained the viable protoplasts, and the other two layers contained the non-viable protoplasts and debris. The top layer of CPW13MAN which remained clear and did not contain any cellular debris or protoplasts was carefully removed without disturbing the band (removing the top layer of CPW13MAN prior to removal of the band minimizes the amount of debris removed with the band) and placed in a second sterile tube. The band was then carefully removed from the top, being careful to avoid taking any of the cloudy layer of debris and transferred to the tube containing the CPW13MAN and gently resuspended (Frearson et al., 1973; Schell, 1991). The protoplasts were pelleted by centrufuging for 5 minutes at 500 rpm (75 x g) and the supernatant removed. The cells were then resuspended to a volume of approximately 10 times the volume of the pellet with 0.7 M BH3. This corresponds to a density of approximately 1 X 10^6 protoplasts/ml and can be confirmed by counting using a haemocytometer.

At this point the protoplasts were ready to be used for the transformation experiments. First, 10-40 plates (10mm X 60mm) were laid out and 3 drops of the protoplasts mixture was placed in the center of each plate. Of these plates, 3-4 were used as control and are treated the same as the other plates except no plasmid or other DNA was added. To the other plates, 10-20 μl (10-20 μg) of plasmid DNA was added to the protoplast drop. To this was added 4 drops of PEG solution (see Appendix

A), and the cells were allowed to stand for 10 minutes. To this mixture was then added 4 drops of a 9:1 v:v solution of Solution A:Solution B (see Appendix A) around the edge, and the cells incubated for another 20 minutes. Finally 15 drops of BH3 were added and the cells then incubated another 10 minutes.

The liquid was then pipetted off very carefully, another 15 drops of BH3 added, the cells allowed to stand another 5 minutes and the liquid again removed carefully. This was repeated twice more to ensure that the cells were adequately washed and the PEG removed. The cells were then suspended in 1 ml of a 1:1 mixture of 0.6 M BH3 and 0.6 M EME (see Appendix) and swirled to spread the medium thinly across the whole plate. The plates were then wrapped with a double layer of Nesco film, placed in a cardboard box, and incubated in the dark at 28°C.

Culturing and Regeneration of Treated Protoplasts

Regeneration of plants from treated protoplasts requires close observation as the cells grow. Not doing this may result in the cells growing too fast and losing their embryogenic capacity. If this happens it is virtually impossible to recover any plants, as rapid cell growth inhibits somatic embryo induction. The procedure for culturing and regenerating the treated protoplasts was as described in Schell (1991).

Testing for Transformed Plants

Polymerase Chain Reaction for Detection of Transformants

The polymerase chain reaction (PCR) was used to initially screen the regenerated 'Nova' tangelo and 'Rohde Red Valencia' sweet orange plants. Since the procedure used was directly related to the results obtained, the discussion of the procedures, primers used, PCR profile and other elements of the PCR procedure will be discussed in the Results and Discussion section.

Testing for Transformed 'Rohde Red Valencia' Sweet Orange Using Dot Blot Analysis

Isolation of DNA from Citrus Leaves for Dot Blot Analysis (Dellaporta et al., 1983, as modified by R. Durham, pers. comm.; Sambrook et al., 1989; Manning, 1991)

Approximately 0.75 to 1.00 g of young leaf tissue was ground in liquid nitrogen using a mortar and pestle. The powder was then transferred to a 50 ml Oak Ridge centrifuge tube and placed on ice. After the nitrogen had evaporated, 15 ml of ice cold DNA extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM Na_2EDTA pH 8.0, 500 mM NaCl, 10mM Beta-mercaptoethanol) and 2 ml of 10% SDS were added. The tube was capped and shaken vigorously, then placed in a water bath at 65°C for 10 minutes.

The tubes were then removed from the water bath and 5 ml of 3 M potassium acetate solution (60 ml/100 ml 5 M potassium acetate, 11.5ml/100 ml glacial acetic acid, 28.5 ml/100 ml H_2O) were added. The tubes were again capped, shaken vigorously, and incubated on ice for 20 minutes. The

samples were then centrifuged at 25,000 X g for 30 minutes. The supernatant was filtered through a miracloth filter and into a clean 50 ml Oak Ridge tube. To this was added 10 ml of isopropanol and the samples allowed to sit at room temperature for 30 minutes or longer. At this point a gelatinous polysaccharide material precipitated with the DNA. This polysaccharide material did not appear to inhibit enzyme digestion but made the final DNA solution very viscous. This could be partially overcome by heating the DNA to 65°C prior to pipetting the samples for digestion.

The DNA was pelleted by centrifugation at 20,000 X g for 30 minutes. The supernatant was poured off and the pellet lightly dried by inverting the tubes on a paper towel for 10 to 15 minutes. The DNA was then resuspended in 0.7 ml of Buffer B (50 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA pH 8.0) and transferred to a 1.5 ml microfuge tube, then centrifuged for 10 minutes in a microfuge to remove any debris. The supernatant was then transferred to another 1.5 ml microfuge tube.

To the DNA solution was then added 0.7 ml of Tris-equilibrated phenol, and the solutions mixed by inverting the tube several times. The samples were then microfuged for two minutes followed by extracting the aqueous upper phase and transferring it to a clean microfuge tube. This was repeated using 0.7 ml of chloroform/iso-amyl alcohol (24:1).

The resulting DNA extract contains a large amount of polysaccharides which can be a problem when resuspending the DNA after ethanol precipitation in a small quantity of TE buffer or water. The extract can be very viscous and difficult to pipet unless diluted. To remove the polysaccharides so as to sufficiently concentrate the DNA for further uses

a differential precipitation with 2-butoxyethanol (ethylene glycol monobutyl ether) was performed (Manning, 1991). The solution obtained after phenol/chloroform extraction was supplemented with 100 µl of a 1.0 M acetate solution (pH 6.5) and 0.4 volumes (250 µl) of 2-Butoxyethanol. The samples were vortexed and placed on ice for 30 minutes (The polysaccharides are visible as a whitish cloud). The samples were then centrifuged in a microfuge for 30 minutes. The supernatant was then removed and transferred to a fresh tube (the polysaccharides remain as a pellet in the bottom of the tube). The volume was then brought up to one volume of 2-butoxyethanol by the addition of another 400 µl 2-butoxyethanol, vortexed and again placed on ice for 30 minutes. The samples were again microfuged for 30 minutes, then the supernatant was decanted off. The resulting DNA pellet was then washed by the addition of 500 µl of 95% ethanol. The samples were allowed to stand at -20°C for at least 30 minutes. At this point the DNA pellet becomes visible as a white pellet in the bottom of the tube. The samples were then centrifuged for 10 minutes and the supernatant decanted off. A final wash with the addition of 500 µl of 70% ethanol followed by microfuging for 5 minutes was then performed. The supernatant was decanted off, the tubes inverted on a kimwipe to remove excess alcohol, and then vacuum dried in a dessicator. The pellet was then suspended in 50 µl of TE buffer and quantified by spectrophometry. An O.D. at 260nm of 1.0 is equivalent to 50 µg/ml of DNA. The quality of the DNA was checked by determining the O.D. 260 to O.D. 280 ratio. DNA should have a ratio of 1.8, RNA a ration of 2.0 (Sambrook et al., 1989). Alternatively the DNA was quantified by agarose gel electrophoresis using lambda DNA of known quantities and

visually estimating DNA content in the samples. The DNA was then placed in the -80°C freezer until needed and thawed on ice prior to use.

Preliminary Screening Using DNA Dot Blots (from instructions supplied with the Amersham Hybond N+ nylon membrane)

Five µl of the sample from the DNA extraction just described was removed, placed in a 1.5 ml microfuge tube, and placed on ice. The samples were boiled for 10 minutes and quick chilled on ice to denature the DNA. To each tube was added 5 µl of 20X SSC (see Appendix B), the samples spun briefly in the microfuge to collect all liquid at the bottom, and placed back on ice. A nylon membrane was wet thoroughly in water, then soaked in 6 X SSC and allowed to dry. The entire contents of each sample were then spotted on the membranes 2.5 µl at a time. The DNA was then cross linked using a DNA cross linker (1200 joules). The membrane was then used for hybridization using radioactive probes as described later.

Confirmation of Transformation

Isolation of DNA for Southern Blot Hybridization (Duran-Vila et al.,1986; Marais et al., 1995)

Prior to isolation of nucleic acids, all labware was sterilized by autoclaving for one hour at 15 psi and dried in the drying oven. All buffers and solutions were sterilized by autoclaving. Gloves were used to handle all glassware during isolation.

For each sample, 5 g of tissue were ground in liquid nitrogen and transferred to a labeled 100 ml beaker containing 10 mls of extraction

buffer (0.4 M Tris-HCl (pH 8.9), 1 % SDS, 0.005 M EDTA (pH 8.0)) and 10 mls of phenol-Chloroform-isoamyl alcohol (12:12:1) and stirred on a stir plate. After all samples were ground they were stirred for an additional 20 minutes, transferred to a 50 ml centrifuge tube, capped and centrifuged at 12,000 RPM for 20 minutes in a JA-20 rotor. The top layer of liquid was removed and placed in another 50 ml centrifuge tube and 3 mls of 3 M sodium acetate (pH 5.5) and 30 mls of cold 95% ethanol added. Samples were incubated for 30 minutes at -80°C and then centrifuged as before. The supernatant was decanted and the tube containing the pellet air dried inverted on several layers of paper towels. The pellet was resuspended in 3 mls of TKM buffer (0.01 M Tris-HCl (pH 7.4), 0.01 M KCl, 0.0001 M $MgCl_2$). The samples were then placed in dialysis tubing (6000-8000 MWCO) which had been boiled in 1 %SDS, 10mM EDTA (pH 8.0) and dialyzed overnight in 1 L of TKM buffer on a magnetic stirrer with gentle stirring at 4°C. The samples were then removed from the dialysis tubing using a pasteur pipet, transferred to 50 ml centrifuge tubes and 3 mls of 4 M LiCL added. Samples were gently mixed and incubated for 4 hours to overnight at 4°C. Samples were then centrifuged for 20 minutes at 12,000 RPM in a JA-20 rotor. The supernatant which contains the DNA was transferred to another 50 ml centrifuge tube and 18 mls of cold 95% ethanol added. Samples were placed at -80°C for 30 minutes and centrifuged as before. The supernatant was discarded, the pellet air dried and resuspended in 100 μl of sterile ddH_2O. The pellet from the LiCl precipitation, which contains ssRNA, was dried and resuspended in 100 μl of sterile double distilled H_2O (ddH_2O). The samples (2 μl of each, with 8 μl H_2O and 3 μl of loading buffer (40% sucrose, 0.25% bromphenol blue) were then ran on a 1% agarose gel in TBE

(see Appendix B) at 100V for 1 hour to quantify the DNA (against Lambda DNA of known concentrations), and to confirm that the desired nucleic acids were in the proper fraction.

Southern Transfer of DNA Isolated from Transformed Plants (Sambrook et al., 1989; R.F. Lee, pers. comm.).

Southern analysis is routinely used to prove that the target DNA has been incorporated into the plant genome. The following procedures were used to test plants which were positive in the preliminary testing by dot blot or PCR assay.

Transfer under neutral conditions.

DNA was isolated from plants to be tested by the methods described previously. At least one non-transformed (never exposed to the plasmid or virus) plant was used as a control in every analysis. A sample of each DNA to be analyzed (around 10-20 μg as determined by quantification) was removed and placed in a separate 0.5 ml microfuge tube. The DNA was then restriction digested by adding 2 μl/10 μl of 10 X OPA enzyme buffer (supplied by Pharmacia with the restriction enzymes) and 10-15 units/sample of restriction enzyme(s) (BglII with or without EcoR1) and incubated for 3 hours to overnight at 37°C. The samples were run on a gel containing 0.5 X TBE (see Appendix B) and 1% agarose in 0.5 X TBE buffer. The gel was then stained with 10 μl of ethidium bromide solution (10 mg/ml) in approximately 100 ml of distilled H_2O for a minimum of 30 minutes, and then destained for 10 minutes in distilled H_2O. The gel was

then observed on the UV light box, photographed (if desired), and the unused portions of the gel trimmed away.

The DNA was nicked by depurination in several volumes of 0.2 N HCl for 10 minutes, followed by a brief rinse in distilled H_2O. The gel was denatured by soaking the gel in several volumes of denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 minutes with constant gentle agitation on a shaker. The gel was then rinsed for a few minutes in distilled H_20. The gel was neutralized by soaking under gentle agitation in several volumes of neutralizing solution (1 M Tris pH 8.0, 1.5 M NaCl) for 15 minutes. The neutralizing solution was then discarded and replaced with fresh denaturing solution and allowed to soak under gentle agitation for another 15 minutes. This was repeated twice more.

While the gel was in the neutralizing solution a piece of Whatman 3MM paper was wrapped around a piece of plexiglass or a stack of glass plates to form a support that was longer and wider than the gel. This was placed in a large baking dish. The dish was then filled to just below the rim of the top support with 10 X SSC (see Appendix B). After the paper was thoroughly wetted, the air bubbles were smoothed out using a glass rod. A piece of nylon membrane (Hybond N+) was cut to the size of the gel and placed in a dish of distilled water until the membrane was wetted completely. The membrane was then immersed in transfer buffer (10 X SSC) for at least five minutes.

After neutralization was complete, the gel was removed, inverted so that the gel was upside down and placed in the center of the wet filter paper. All air bubbles were carefully removed. The wet nylon membrane was than carefully placed on top of the gel and one corner cut to use as

a reference point. Once the membrane was placed on the gel it was not moved. All air bubbles were again carefully removed so as not to interfere with DNA transfer. Two pieces of 3MM paper were then cut to the same size as the gel, wet with 2 X SSC, and placed on top of the nylon membrane. The air bubbles were once again removed.

A stack of newspaper 3-5 cm high were cut just smaller than the 3MM papers and placed on top of the 3MM papers. A glass plate was placed on top of the stack and weighed down with a 500 g weight. The objective was to set up a flow of liquid from the reservoir up through the gel and the membrane and into the stack of newspaper. The DNA fragments should be eluted from the gel and deposited onto the membrane. The transfer was allowed to proceed overnight (8-24 hours).

After the transfer was completed, the newspaper and the 3MM papers above the gel were removed. The gel, with the membrane still in place, was turned over onto a dry sheet of 3MM paper and the positions of the gel slots marked with a very soft lead pencil. The gel was then peeled away and discarded. The membrane was soaked in 6 X SSC for 5-15 minutes with gentle agitation to remove any pieces of agarose sticking to the membrane. The membrane was removed and dried on a fresh piece of 3MM paper for 30-60 minutes. The DNA was then ready to be hybridized with a labeled probe.

Transfer under alkaline conditions.

The DNA extraction through electrophoresis and photographing of the gel was performed in the same manner as for transfer under neutral conditions. After electrophoresis the DNA in the gel was nicked by placing the gel in 0.25 N HCl for 15-20 minutes. The gel was then soaked

in 0.4 N NaOH for 30-45 minutes. At the same time the transfer system was set up as for transfer using neutral conditions, but with the 10X SSC replaced by 0.4 N NaOH. The transfer was allowed to continue overnight. The membrane was removed and the lanes marked with a red pen. The membrane was soaked with gentle agitation in 5X SSC to neutralize, then air dried for a few minutes. Many protocols call for baking the membrane at 80°C for two hours or UV cross-linking to fix the DNA to the membrane, but this is not necessary for positively charged membranes. The membrane was then placed in a plastic bag and was ready for hybridization.

Preparation of Radioactive Probes by Random Primer for Southern Blot Hybridization

The probe used for Southern blot hybridization was prepared using PCR. The coat protein gene of T30 and T36 were amplified using primers C104 (CCTACTCGAGATTAACGTGTGTTGAATTTCC, 3' neg. sense) and C118 (AACTGCAGCGGCCGCCGACGACGAAACAAAG,5' plus sense)(obtained from J. Castillo). The PCR reaction was performed in capillary tubes using a profile with an initial denaturation at 94°C for 15 sec. followed by 30 cycles of denaturation at 94°C for 5 sec., annealing at 50°C for 10 sec., extension at 73°C for 20 sec., followed by a final extension at 72°C for 5 minutes. Samples were then ran on a 1 % agarose gel (using low melting point agarose in 0.5 X TBE), and the band of the appropriate size (approx. 670 bp) was excised from the gel and placed in a 1.5 ml microfuge tube. Five volumes of TE buffer were added and the samples incubated at 65°C for ten minutes or until the agarose was fully melted. If the volume was more than 750 µl, the samples were subdivided and then an equal volume of tris-

equilibrated phenol was added. Samples were vortexed, than centrifuged for 3 minutes. The top layer containing the DNA was removed and placed in another microfuge tube. To the sample was then added 0.5 volumes of Tris-equilibrated phenol and 0.5 volumes of chloroform/iso-amyl alcohol (24:1) and the samples vortexed and centrifuged for 3 minutes. The top layer was again removed and transferred to a separate tube and an equal volume of chlorform/iso-amyl alcohol (24:1) was added. Samples were vortexed and centrifuged for 3 minutes and the top layer again removed and transferred to a separate tube. The DNA was ethanol precipitated by the addition of 0.1 volumes on 3M sodium acetate and 3 volumes of 95 % ethanol followed by centrifugation for 15 minutes. The supernatant was decanted and the DNA pellet was then washed by the addition of one volume of 70% ethanol followed by centrifugation for 5 minutes. The supernatant was again decanted and the tube inverted on a Kim Wipe for a few minutes, then placed in a vaccuum dessicator and the DNA dried under vacuum. The DNA was then resuspended in sterile distilled water at an approximate concentration of 25 ng/μl.

The probe was prepared by random primer labeling using the Prime-a Gene Labeling System (Promega Corp.) as per the instructions provided. For each labeling reaction 25 ng of probe DNA was denatured by boiling for 5 minutes and then quick chilling on ice. The DNA was usually placed in nuclease free water to bring the final volume of the reaction up to 50 μl prior to boiling. After denaturation, 10 μl of 5 X buffer, 2 μl of the unlabeled dNTP mix (dATP, dCTP, dGTP), 2μl nuclease free BSA, 5μl [α-^{32}P] dCTP (50 μCI, 3000Ci/mmol), and 1 μl (5 units) DNA Polymerase (Large (Klenow) fragment) were added. The reaction was allowed to proceed for 3

or more hours and the reaction stopped by the addition 2.5 µl of 0.5 M EDTA (pH 8.0), followed by boiling for two minutes and quick chilling prior to use.

Hybridization of DNA With a Labelled Probe. (Sambrook et al., 1989; T. Kamps, pers.comm.)

The nylon membrane prepared previously for either the dot blot analysis or Southern blot hybridization, was placed in a plastic bag and prehybridization solution was added. The prehybridization solution consisted of 6 X SSC, 5 X Denhardts reagent, 0.5 % SDS, and 100 µg/ml salmon testes DNA (boiled 10 minutes and quick chilled). The bag was carefully sealed and placed inside a ziploc bag and incubated for 1-2 hours to overnight (16 hours) in a water bath at 68°C. the probe prepared by Random Priming was added at 5 X 10^5 to 2 X 10^6 CPM after boiling for 3 minutes and quick chilling on ice. The bag was resealed, placed in a ziploc bag, and incubated overnight (16-24 hours) in a water bath at 65°C.

The next morning the hybridization solution was disposed of in the radioactive waste and the membrane was rinsed twice in wash solution (100 ml/l 20 X SSC, 10 ml/l 10% SDS, 200 ml/l of 0.1 M $NaPO_4$, pH 7.2) for 15 minutes each at 65°C with occasional gentle agitation. The membrane was placed in a plastic bag and approximately 50 mls of stringency buffer (5 ml/l 20 X SSC, 10 ml/l 10% SDS, 20 ml/l of Na PO_4 pH 7.2) was added, the bag sealed and incubated in the water bath for 15 minutes at 65°C. The solution was discarded and fresh stringency buffer added and the membrane was incubated in the water bath at 65°C for another 15 minutes.

The membrane was dried on a piece of 3MM paper for a few minutes, then placed on a fresh piece of 3 MM paper. The corners of the membrane were taped down and then wrapped in plastic wrap. The membrane was placed face down on a piece of X-Ray film in a film cassette with an intensifier screen. The cassette was sealed, wrapped in aluminum foil, and placed in the -80°C freezer for 1 to 7 days (depending on the half-life status of the isotope and the quality of the random priming). The film was developed according to the directions provided with the developer.

Southern Blot Hybridization Using the GENIUS Non-radioactive Labeling System (Boehringer-Mannheim)

Prior to hybridization, the labeled DNA to be used as a probe needed to be prepared. The plasmid DNA which was to be used for the probe was first denatured by boiling for 10 minutes followed by quick chilling in ice/alcohol. An aliquot of 1 μg of freshly denatured DNA was removed and placed in a microfuge tube on ice and to this was added 2 μl of the supplied hexanucleotide mixture, and 2 μl of the supplied dNTP labeling mixture. The volume was then brought up to 19 μl with sterile distilled H_2O, 1 μl of Klenow enzyme added and the mixture was incubated for 60 minutes or more at 37°C. The reaction was stopped by adding 2 μl of EDTA solution (0.2 M, pH 8.0).

The labeled DNA was then precipitated by adding 2 μl of LiCl (4M) and 60 μl of cold 100% ethanol. The DNA was left for at least 30 minutes at -70 C, or at least 2 hours at -20 C, and then centrifuged at 12,000 X

g for 10 minutes. The supernatant was then poured off and the pellet washed with cold 70% ethanol and recentrifuged at 12,000 X g for 10 minutes. The alcohol was then poured off and the pellet dried under vacuum. Finally, the DNA was dissolved in 50 µl of TE buffer and stored at -20°C until needed.

Approximately one hour before starting the hybridization, the hybridization solution was prepared (the blocking reagent does not dissolve very rapidly, thus the need to prepare in advance). The hybridization solution consisted of 5X SSC (see Appendix B), 0.5% blocking reagent (supplied), 0.1% N-laurylsarcosine (Na-salt), and 0.02% SDS. It was heated in a 70°C water bath in order to facilitate the blocking reagent going into solution.

The membranes which had been previously prepared (by Southern transfer or dot blotting) were wet briefly with 5X SSC and then placed in a plastic sealable bag. Hybridization solution (20 ml) was added, the bag sealed, and prehybridization carried out at 68°C for 4 hours. The solution was replaced with 25 ml of hybridization solution and 50 µl of labeled DNA. The membranes were incubated overnight (18 hours) at 68°C. The membranes were washed twice, 5 minutes each, with at least 100 ml of wash buffer (2X SSC, 0.1% SDS), followed by two washes of 15 minutes each at 68°C in stringency buffer (0.1X SSC, 0.1% SDS). The membranes were air dried and stored protected until used for the immunological detection using the labeled DNA probe.

The membranes were first washed for 1 minute in buffer 1 (Tris-HCl, 100 mM, NaCl, 150 mM, pH 7.5). Buffer 1 was poured off and 100 ml of buffer 2 (0.5% blocking reagent in Buffer 1) was added and the membranes

incubated for 30 minutes. The membranes were again washed for 1 minute with buffer 1 and placed in a plastic sealable bag. Antibody-conjugate to the labeled probe was diluted to 150 mU/ml (1:5000) in buffer 1, and added to the membranes and incubated another 30 minutes. Unbound antibody-conjugate was removed by washing twice (15 minutes each) with buffer 1. The membranes were equilibrated for 2 minutes in 20 ml of Buffer 3 (Tris-HCl, 100 mM, NaCl, 100 mM, $MgCl_2$, 50 mM, pH 9.5). Freshly prepared color solution (45 μl NBT solution, 35 μl x-phosphate solution in 10 ml of buffer 3) was added and the color precipitate allowed to form overnight in the dark. The reaction was stopped by washing the membranes overnight in TE buffer. The membranes were stored in TE Buffer until they were needed. Alternatively, the membranes could be stored dry, however the color may fade. The color could be enhanced by placing the dry membrane back in TE buffer.

Western Immunoblot analysis for Detection of the Coat Protein

Leaves were collected and kept in the cold room or in the -20°C freezer until needed. The leaves were then ground in liquid nitrogen and the powder transferred to a 1.5 ml microfuge tube. Immediately, 1 ml of 1X treatment buffer (60 mM Tris, 2% SDS, and 1% 2-mercaptoethanol) was added and the sample vortexed. This was repeated until all samples were prepared. The samples were allowed to set for 15-30 minutes, then centrifuged in the microfuge at 10,000 RPM for 10 minutes to pellet the debris. The supernatant was removed and placed in another sterile 1.5 ml microfuge tube. A 100 μl aliquot of the sample was removed and placed in

1.5 ml microfuge tube and frozen until used. The rest of the sample was concentrated using an RC10 concentrator/evaporator (Jouan, Inc) to approximately 1/5 the original volume. The heat setting of around 3.5 was used in order to keep the samples from freezing. Concentration usually was finished in around 2 hours. The volume of the samples was then brought to approximately one fifth the original volume by the addition of additional 1X treatment buffer (all samples were not concentrated equally so the volumes need to be made equal for all the samples) and the samples placed at -20°C until needed.

The proteins were separated on a 14% polyacrylamide gel on a BioRad minigel apparatus. The gel was prepared by adding 4 ml/gel of running gel stock (see Appendix B), 53 µl/gel of 10% TEMED, and 13 µl/gel of 10% ammonium persulfate. The gel was then loaded to a depth of 5 cm using a transfer pipet. This layer was then overlayed with Stacking gel containing 2 ml/gel stacking gel stock (see Appendix B), 40 µl/gel TEMED, and 10 µl/gel of 10% ammonium persulfate. A gel comb was then place in the gel and the gel allowed to polymerize for at least one hour.

While the gel was polymerizing, the protein samples were aliquoted into an microfuge tube and boiled for 10 minutes. After the gel was polymerized the samples (30 µl) were loaded on the gel and the gel was run at 40mA/gel for 105 minutes in SDS tank buffer (1g/l SDS, 14.5 g/l Glycine, 3g/l Tris).

After the gel was finished running, the gel was removed and placed in protein transfer buffer (25 mL Tris-HCl pH 8.3, 192 mM Glycine, 20 % Methanol) for 15-20 minutes. The buffer was then removed and two pieces of Whatmann filter paper cut to the size of the gel was wet with protein

transfer buffer and placed on top of the gel. The gel and filter paper were carefully removed from the tray and trimmed to size. A nylon membrane (Immobilon P from Millipore Corp.) was wet briefly in methanol, the methanol removed by placing the membrane in H_20 until submerged, and soaked briefly in protein transfer buffer. (Alternatively, a nitrocellulose filter wet in transfer buffer can be used.) The membrane was placed on top of the gel and an additional two pieces of Whatmann filter paper wet with protein transfer buffer placed on the membrane, and again trimmed to size. The whole sandwich was placed with the membrane side down on a semidry electroblotter and the proteins transferred by running at $0.4A/cm^2$ for 2 hours.

The membrane was removed and placed in 20 ml TTBS (0.5M Tris-HCl pH 7.5, 2M NaCl, 0.0005% Tween-20) containing either 3% bovine serum albumin (BSA) or 1% gelatin as a blocking agent, and placed on a shaker at 75RPM for at least 20 minutes. The desired primary antibody was added at a 1:10000 v:v dilution and placed back on a shaker for 30-45 minutes (or overnight). The membrane was then washed with TTBS at least 3 times for 10 minutes on a shaker. Fresh TTBS (20 ml) with 1% gelatin or 3% BSA was added along with the appropriate secondary antibody at a dilution of 1:10000 v:v and again placed on shaker for 4-6 hours (or overnight). The membranes were again washed at least 3 times for 10 minutes on shaker with TTBS. The membranes were developed by the addition of a thin layer (approx. 20 ml) of NBT-BCIP substrate solution (12 g/l Tris, 5.8 g/l NaCl, 1 g/l $MgCl_2$, 80 mg/l BCIP (disodium salt), 160 mg/l NBT, pH 9.5). Modifications of this procedure were used and are described as appropriate in the results and discussion.

CHAPTER 4

RESULTS AND DISCUSSION

Plasmid Preparation

The coat protein genes for CTV isolates T30 and T36 were inserted into the plasmids pMON10098 and pBPFΩ-7 by Dr. Niblett's laboratory and supplied in *E. coli* strain DH5α. These plasmids were designated pMON10098-T30, pMON10098-T36, pBPFΩ7-T30, and pBPFΩ7-T36. (Figure 3-3 shows a map of these plasmids.) Testing for correct orientation and coat protein expression were performed in Dr. Niblett's laboratory prior to sending the plasmids. The bacterial cultures were replated and single colonies selected to begin large scale plasmid preparation as described in the materials and methods.

Transformation of Citrus Protoplasts and Plant Regeneration

The success or failure of isolation and transformation of *Citrus* protoplasts and the ultimate regeneration of plants is contingent on the quality of the starting material and quality of the protoplasts after isolation (high yields of viable protoplasts with little debris). *Citrus* suspension cultures

used were chosen because they were exhibiting good regeneration capacity at the time. The best protoplasts were obtained from suspensions 5 to 12 days after subculture when isolated as described in the materials and methods. Many isolation procedures do not use the sucrose/mannitol gradient for purification. However using the gradient allows for the removal of the non-viable protoplasts and cellular debris which could interfere with the uptake of the plasmid by the viable protoplasts, possibly increasing efficiency.

After the protoplasts had been collected from the gradient, they were resuspended in 0.6 M BH3 at a density of approximately 1 X 10^6/ml as determined by visual observation of the pellet. This would usually yield a final protoplast density after the transformation experiments of 5 X 10^5. Transformation experiments and plant regeneration were performed as described in the materials and methods. For each transformation experiment attempted, 20 to 30 plates were used. Cell wall regeneration was observed within 24 to 48 hours, and cell divisions began after approximately three days. Colonies (microcalli) begin to form in as little as two to three weeks. It was found to be critical to not let the colonies develop too fast prior to osmoticum reduction and the eventual plating of the developing microcolonies onto solid EME medium (see Appendix A). With extremely rapid colony development, microcalli tended to lose their embryogenic capacity, thereby inhibiting embryo and plant recovery.

Generally, the colonies were ready to be transferred to solid EME in 4 to 8 weeks. Shortly thereafter embryos began to form. These embryos were removed and transferred to another fresh plate containing EME, and

allowed to proliferate. Embryos were then transferred to 1500 medium (see Appendix A) for further growth, and finally to B+ medium (see Appendix A) for germination. After the formation of plantlets, the plantlets were removed and placed on rooting medium (see Appendix A) until roots formed and then subsequently transferred to soil.

The time from initial protoplast isolation to plant establishment in soil was quite variable. Several factors influence this chain of events. Delays in the osmoticum reduction process and in transferring slowed down the growth. In some cases the developing embryos could be transferred directly from EME to B+ medium, skipping the transfer to 1500 medium. At other times they may need an additional pass on EME, 1500, or B+ media. However, if all went smoothly plantlets were established in soil in as little as 5 to 6 months.

Over 40 transformation experiments were attempted over the duration of this project. These experiments were done with a variety of scion cultures using all four of the plasmids. The reasons for the lack of plants regenerated from many of the experiments can generally be traced to problems related to the culturing of the protoplasts, or to the condition of the suspension cultures used for protoplast isolation. If large numbers of microcalli are produced (i.e. high plating efficiency) there is a tendency for callus to be formed at the expense of the production of embryos. If embryos do form there is often an overgrowth of these embryos by the callus and the result is no further development of the embryos. This is the biggest factor contributing to the lack of plant regeneration in many of the transformation experiments. Other reasons for the failure of plant regeneration include contamination of the media, the condition of

the protoplasts after isolation and transformation, and other undetermined factors. For a detailed description and photograph of the developmental sequence for protoplasts see Schell (1991).

As a result of these transformation experiments numerous regenerated plants were obtained. Using the plasmid pMON10098-T30, 59 'Nova' tangelo plants were obtained. Using the plasmid pMON10098-T36, 12 'Valencia' sweet orange plants were obtained. Using the plasmid pBPFΩ7-T36, 500 'Rohde Red Valencia' sweet orange plants were obtained. Several other attempts were made to transform these and other scion varieties, with the four plasmids but were unsuccessful due to difficulties in isolation, culture, and regeneration. These difficulties are the limiting factors in transformation by direct DNA uptake.

The number of plants recovered per embryo was not tracked due to the amount of time necessary to do this. As a result, there is a possibility that some of the individual plants may have come from the same embryo. Multiple shoots from a single embryo were observed in these experiments.

Screening for Transformants

Selection During the Early Stages of Protoplast Growth and Microcalli Development

The stage at which selection is performed is dependent on the method of transformation and the plasmid used. Using *Agrobacterium* as a vector for transformation of plant tissues, selection is commonly done immediately after co-cultivation utilizing the antibiotic resistance marker contained in the plasmid. This has also been done with protoplasts

transformed by direct DNA uptake (Davey et al., 1989). However, there are problems with immediate selection for transformed protoplasts using the antibiotic resistance conferred by the plasmid. First, plating density plays a major role in regeneration and survival of protoplasts. Kao and Michayluk (1975) developed a medium (K8P) for low density plating of *Vicia hajastana* protoplasts which has been commonly used in other systems. This is the medium from which the BH3 medium used in this study was derived (Grosser, 1994). However no studies have been performed to determine the lowest concentration at which *Citrus* protoplasts can survive. Some labs have attempted to circumvent this problem by delaying the placement of the protoplasts on antibiotic-containing medium for a period of time (Paskowski and Saul, 1988). Secondly, if selection is done at the protoplast level the effect of the dead protoplasts has to be considered. If transformation efficiency is as good as 1%, then 99% of the protoplasts would die under selection. These dying protoplasts could release many toxic compounds into the medium which would adversely effect the development and survival of those that are antibiotic resistant (or putative transformants). Therefore selection at the protoplast level was not a practical idea for *Citrus*.

The second most likely stage for selection is after the formation of microcalli or calli. Experiments were conducted to test whether or not the microcalli could survive on medium containing the antibiotic geneticin. Resistance to geneticin is controlled by the NPTII gene contained in both plasmids. Microcalli from transformation experiments using 'Rohde Red Valencia' sweet orange, 'Nova' tangelo and 'Valencia' sweet orange were placed on EME media containing 0, 5, 10, 20 and 50 μg/ml

of geneticin. All of the microcalli died on levels of 20 and 50 μg/ml and little or no death was noted in the lower two levels of 0 and 5 μg/ml. The reaction at 10 μg/ml was mixed with either the whole plate of microcalli dying or the whole plate of microcalli surviving. No selection seemed to occur at any antibiotic concentration. No embryos developed from any of the surviving microcalli. It appeared that some sort of interaction between the microcolonies may have been taking place which was interfering with any selection processes that may have been occurring. Repeated experiments yielded the same results. The fact that the antibiotic was in the medium may also have been playing a role. To test this, experiments were also conducted placing the geneticin at levels of 0, 2.5, 5 and 10 μg/ml in liquid EME, which was layered over the solid EME at the rate of 1 ml/ plate (60mm X 10mm). Microcalli were placed in this liquid EME. Death occurred at 10 μg/ml, some of the plates showed no effect at 5 μg/ml while others showed total death of the microcalli. There seemed to be variable survival in some of the plates at 2.5 μg/ml, ranging from death of the microcalli to total survival. However none of the surviving microcalli were able to form embryos. (It should be noted that these microcalli were from the same transformation experiments that eventually gave rise to the transgenics reported herein). Because of the wide variation in callus survival rates, and the lack of repeatability, it was concluded that selection with geneticin at this stage was not the best selection procedure.

It is quite common to use a scoreable marker such as the histochemical GUS assay for the detection of the β-glucuronidase product of the uidA (GusA) gene (Jefferson et al., 1986; Jefferson, 1987;

Jefferson et al., 1987; Jefferson, 1989). This scoreable marker can be used for selection by performing the assay on a sample of plant tissues such as a leaf piece. However, the plasmids that were supplied did not contain the uidA gene, thus selection by this method was not an option. Selection using neomycin phosphotransferase (NPTII), was not performed as the assays are tedious and relatively difficult, in addition to being susceptible to various endogenous activities in the plant cells, which limits the sensitivity (Reiss et al., 1984). Recently, easier and cheaper ELISA procedures for NPTII selection have become available from many commercial companies, but these were not available at the time these experiments were performed.

Polymerase Chain Reaction for Selection of Transformants

Testing of 'Nova' tangelo and 'Valencia' sweet orange regenerants

The first plants regenerated were 59 'Nova' tangelo from experiments using plasmid pMON10098-T30, and 12 'Valencia' sweet orange from experiments using plasmid pMON10098-T36. Since the objective of this study was the production of plants containing the coat protein gene, and not in the development of an improved transformation procedure, data was not taken to determine the percentage of protoplasts regenerated into plants. It was determined that time was better spent in obtaining the largest number of plants possible, rather then in the tedious chore of cataloging the development of individual protoplasts and microcolonies.

These plants were tested by using a pooled polymerase chain reaction (PCR) in which the samples were pooled together in groups of 5 (and one of

6) prior to DNA isolation. Isolation of DNA (see materials and methods) is the most time consuming step in the PCR process and pooling the samples in the initial screening process allows the test to be performed much quicker. Also, the cost of all reagents can be greatly reduced. The members of each positive pool could then be retested individually and sorted out from the pool. The PCR reactions were carried out using a Precision Scientific GTC1 Thermal Cycler. This is a water cooled (as opposed to Peltier-effect cooled) machine. The PCR profile was an initial 2 minute denaturing at 94°C, followed by 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 55°C, 1 minute extension at 72°C, and then ending with a 10 minute final extension at 72°C. The reaction was carried out in 1X Promega Taq Polymerase reaction buffer, 25μm $MgCl_2$, 200 nmol of each primer and 1 unit of Taq polymerase per sample. The T30 infected control also contained 0.001M DTT, 1 μl RNasin, and 1 unit AMV reverse transcriptase and was incubated at 42 °C for 45 minutes prior to amplification. Final reaction volume was 50 μl, and the samples were overlayed with 2 drops of sterile mineral oil. The primers were JC-10 (5'-GAATTCGCGGCCGCTCAACGTGTGTTAAATTTCC) and JC-11 (5'-AGATCTACCATGGACGACGAAACAAAG), specific to the coat protein gene of isolates T36 and T30. The final PCR product was run on a 1% Agarose Gel in TBE.

After performing the PCR on the pooled samples from the 59 'Nova' tangelos and 12 'Valencia' sweet orange regenerants, one pool was identified as containing at least one positive sample. DNA from the individual plants represented in this pool (containing samples N29, N30, N32, N33, and N34 where the N signifies a 'Nova' tangelo plant) was then

isolated and the PCR repeated as before. From this PCR, 3 plants (N32, N33, N34) were identified as being PCR positive for the coat protein gene (Figure 4-1). This result was repeatable. These plants were repotted and placed in the greenhouse to allow for adequate leaf growth for Southern analysis.

Testing of the 'Rohde Red Valencia' sweet orange regenerants

The 'Rohde Red Valencia' sweet orange plants were produced later than the other two varieties described in the previous section. A new MJ Research PTC-100 thermal cycler was purchased and used for testing these plants. This machine is cooled by the Peltier effect and reactions are carried out in 96 well plates, as opposed to the 0.5 ml tubes used with the old machine. Initial experiments were performed using the method described in the previous section except that the Idaho Technology medium buffer was substituted for the Promega buffer A, and the final reaction volume was 25 μl/reaction. The PCR profile was as follows: an initial 2 minute denaturation at 93°C; 35 cycles of 1 minute denaturation at 93°C, 30 seconds annealing at 55°C, 30 seconds extension at 72°C; and a 5 minute final extension at 72°C.

While screening these plants 'false positives' began to be produced. These 'false positives' would appear in all samples including the non-transformed controls and sometimes the buffer controls. The bands were of the same size (approx. 700 bp) as expected for the CTV coat protein gene which we were trying to detect. The appearance of the bands, however, differed in that the band was diffuse rather than a clear distinct band as should be obtained. Even more problematic, these false

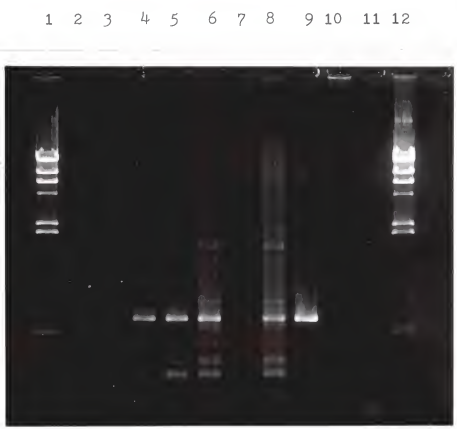


Figure 4-1. Results of the PCR reaction of the samples contained in the positive pool. Lanes 1 and 12 are Lambda HindIII markers. The arrow points to the marker for 564 bp. Lane 10 is blank, Lane 11-- buffer control. Lane 2-- N29, Lane 3-- N30, Lane 4-- N32, Lane 5-- N33, Lane 6-- N34, Lane 7-- untransformed control plant, Lane 8-- a T30 infected plant, and Lane 9-- the plasmid control.

positives did not manifest themselves all the time, particularly in the buffer control. As a result, we had to take a step backwards and try to determine the source of this problem, and how to alleviate it.

The initial inclination was to blame the problem on the primers, specifically the primers JC-10 and JC-11. These primers were originally used by H. R. Pappu (Dr. Niblett's Lab) to isolate and clone the CTV coat protein gene from isolate T36, which was then inserted into the plasmids that were used for transformation. Closer examination of the primers revealed the possibility of a hairpin structure that could occur with primer JC-10 (Figure 4-2) because there is a sequence of 4 bases at the ends of the primer which can align with each other and anneal. This hairpin structure could contribute to mispriming and result in false positives. Onesolution obtained from the Molecular Biology usergroup on the Internet was to denature the primers by boiling and quick chilling them prior to adding them to the reaction mix. This was attempted and seemed to work the first time; however, in subsequent experiments, the same variability was evident. Consequently, new primers were obtained or designed in an attempt to circumvent this problem (Figure 4-3). The primers 728 and 729 are used to amplify a portion of the CaMV 35 S promoter, which should have been inserted into the genome of the transformants along with the coat protein gene. The other primers were specific to various segments of the coat protein gene. Despite using different primers, 'false positives' continued to be an intermittent problem. Occasionally bands would be found for only a few of the samples, indicating a true result (that is-- no 'false positives'), but the results were not repeatable.

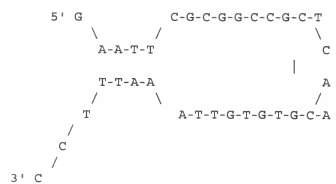


Figure 4-2. A Schematic diagram of how the Primer JC-10 can form a secondary hairpin structure.

C-10 (equivalent to CN120 of C. L. Niblett)

5'- GAA TTC GCG GCC GCT CAA CGT GTG TTA AAT TTC C

C-11 (equivalent to CN119 of C. L. Niblett)

5'- AGA TCT ACC ATG GAC GAC GAA ACA AAG

Eli-1 (CTV-CP, nt 116-139, seq. from isolate T-36)

5'- ACG ATG TTG TTG CTG CCG AGT CTT

Eli-2 (CTV-CP, nt 577-600, seq. from isolate T-36)

5'- CAA GAA ATC CGC ACA CAA GTA ATG

JS01 (CTV-CP)

5'- CCG ACT ACT ACA GCA TCA ATG

JS02 (CTV-CP)

5'- ATG GAC GAC GAA ACA AAC

CN85 (CTV-CP, internal primer)

5'- AAC GTA GCA TCG GGA GGG -3'

CN86 (CTV-CP, internal primer)

5'- CGG TTA CCA ATA CCC TTA G -3'

728 (CaMV 35 S PROMOTER)

5'- GAT ACA GTC TCA GAA GAC C -3'

729 (CaMV 35 S PROMOTER)

5'- CCT CTC CAA ATG AAA TGA AC -3'

Figure 4-3. PCR primers used for testing regenerated plants for integration of the coat protein gene or CaMV 35 S promoter gene.

Other parameters were looked at as the source of the problem. These included changing the reaction buffer, magnesium chloride concentration, addition of bovine serum albumin, and lowering the DNA concentration. The same variable results were obtained. Similarly, experiments with E. S. Louzada looking at whether the concentration of the primers was responsible for the 'false positive' results were also inconclusive. In addition, changes in the PCR reaction profile involving the annealing temperature were also unsuccessful.

As a result of these experiments the conclusion was drawn that these 'false positives' were the result of contamination in the laboratory and/or equipment. Without significant expenditure to purchase PCR dedicated equipment to use throughout the procedure this problem could not be overcome. Because of this, screening by PCR was not a reliable technique to screen for transformants, and another more reliable technique had to be found.

Screening For Transformants Using Dot Blot Analysis

Although there were no recent reports in the literature of the use of a pooled dot blot analysis for the detection of transformants, the decision was made to see if it would be possible to select transformants in this manner. The approach used was similar to that used with the PCR screening, using pools of five samples in the initial screen.

DNA isolation and dot blot analysis were carried out as described in the materials and methods section. From the 90 pools tested, 23 pools showed a postive or inconclusive result. DNA from the members of these

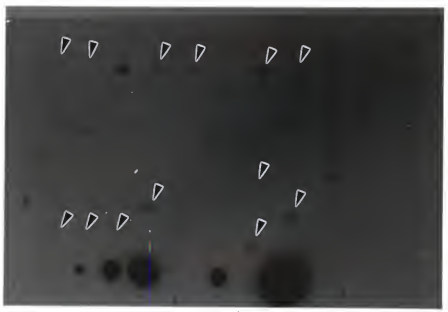
pools was isolated and the procedure was repeated with these individuals. From these blots 22 positive individuals were identified, representing 11 of the original 23 pools (figure 4-4). These positive plants were identified as R73, R82, R83, R121, R124, R129, R140, R142, R146, R147, R149, R150, R152, R153, R268, R269, R270,R271, R271b, R272, R276, R328, and R368. The plant R368 died shortly after the Southern dot blot analysis. The large size of the plasmid control blots is due to the amount of sample in the blot. All trees testing positive were repotted and saved for further analysis.

Confirmation of Transformation

Southern Hybridization Analysis Using the Non-radioactive GENIUS System

In order to confirm transformation, Southern hybridization analysis of the PCR product of the 'Nova' tangelos was performed. This analysis was performed using the GENIUS non-radioactive system as described in the materials and methods. Using this method, all 3 'Nova'tangelos were shown to be transformed. Figure 4-5 shows the results of the Southern hybridization analysis experiment. In this experiment the lower band corresponds to the coat protein gene, while the higher molecular weight bands are probably the result of duplexes being amplified in the PCR reaction.

Figure 4-4. Dot blot hybridization screening for transformed 'Rohde Red Valencia' sweet orange. Arrows point to the samples identified as positive. Starting from the upper right hand corner of the top autoradiograph, the positive plants are R146, R147, R149, R150, R152, R153, R276, R328, R124, R129, R140, R142, and R271b. From the upper right corner of the bottom autoradiograph the samples are R121, R73, R82, R83, R268, R269, R270, R271, R272, and R368. The overexposed dark dots on the bottom of each blot are plasmid controls.



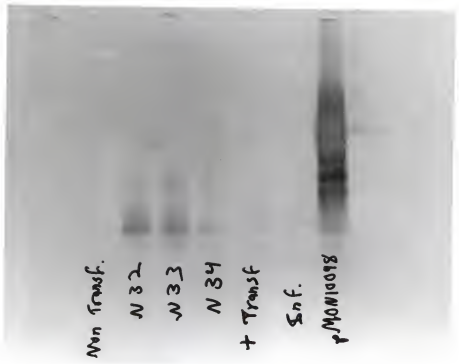


Figure 4-5. Southern hybridization using the GENIUS Non-Radioactive System with 'Nova' tangelo plants found to be transformed by PCR analysis. Lane 1 is a non-transformed control plant Lanes 2-4 are transformants N32, N33 and N34 respectively. Lane 5 is a confirmed transformed Carrizo (from G. Moore). Lane 6 is a T30 infected plant. Lane 7 is the plasmid control. The probe was made to the plasmid pMON10098-T30.

Southern Hybridization Analysis Using Radioactive Probes

Initial experiments using DNA isolated by the method used for dot blot analysis were not successful. Efficient cutting of the DNA was difficult to achieve. Changing from the Promega restriction enzymes to enzymes purchased from Pharmacia Co. improved the efficiency, but the digestions were still not complete. Consequently, it was concluded that the problem was possibly due to the quality of the DNA isolated. To improve the DNA quality and subsequent restriction digest, a new DNA isolation procedure was used (see materials and methods). This procedure was initially developed to isolate viroids from infected tissue (Marais et al., 1995), but it was also efficient at isolating total genomic DNA from the plant tissue. The method achieved the isolation of high yields of high molecular weight DNA (up to 10 µg/g of fresh leaf tissue). This DNA could be very efficiently digested with the Pharmacia Co. restriction enzymes. Using DNA isolated in this manner, Southern hybridization analysis was performed on the putative transformants. The genomic DNA was restricted with the restriction enzymes EcoRI and BglII to cut out the coat protein gene. The results obtained with the 'Nova' tangelo plants are shown in figure 4-6. The DNA was probed using a ^{32}P labeled probe to the plasmid pMON10098-T30. The expected band of 670 bp for the coat protein gene was obtained. The Southern hybridization analysis confirms that the 'Nova' tangelo plants N32, N33, and N34 were transformed with the coat protein gene of isolate T30.

Southern hybridization analysis was also performed on the healthiest of the 'Rohde Red Valencia' sweet orange plants which were positive by the

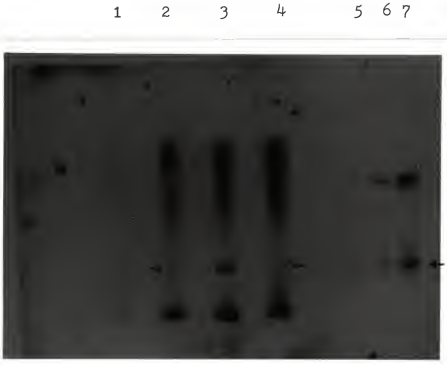
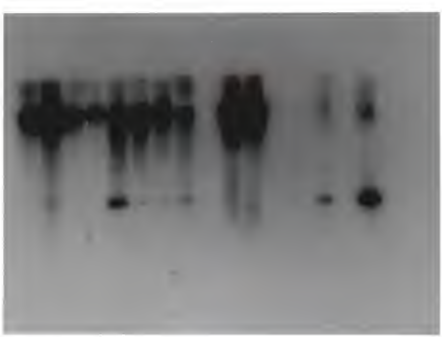


Figure 4-6. Southern hybridization of the 'Nova' tangelo transformants using a radioactive probe to the coat protein gene that was prepared by PCR (see text). Lane 1 is a nontransformed 'Nova' tangelo. Lane 2 is N32, lane 3 is N33, and Lane 4 is N34. Lanes 5-7 are plasmid controls of 0.00001, 0.0001, and 0.001 μg. The arrows point to the coat protein gene band. The bands for N32 and N34 are very faint and may not be visible in the photograph, but are visible on the original autoradiograph. The top band in the plasmid lanes is undigested plasmid and the visible smear in the other lanes is the result on incomplete digestion of the DNA.

Figure 4-7. Southern hybridization using a radioactive probe of the transformed 'Rohde Red Valencia' sweet orange. In the upper autoradiograph, lanes 1-8 are the transformants R121, R124, R129, R140, R146, R150, R152, and R153. Lanes 9 and 10 are nontransformed 'Valencia' controls. Lanes 11-13 are plasmid controls of 0.00001, 0.0001, and 0.001 μg. In the lower autoradiograph lanes 108 are the transformants R268, R269, R270, R271, R271 (second sample), R271b, R272, and R276. Lanes 9 and 10 are nontransformed 'Valencia' controls. Lanes 11-13 are plasmid controls of 0.00001, 0.0001, and 0.001 μg. The background is due to incomplete cutting of the genomic DNA and the large amount of DNA present.

1 2 3 4 5 6 7 8 v v p p p



1 2 3 4 5 6 7 8 v v p p p

dot blot hybridization (figure 4-7). Not all of the plants could be analyzed due to their poor growth and lack of leaf material.

This lack of growth could be due to a positional effect in regards to the location of insertion of the coat protein gene in the DNA of the plant. The large amount of background in the Valencia nontransformed controls is due to the accidental loading of twice the quantity of sample as for the putative transformants. Despite the increased amount of sample, no band appeared corresponding to the coat protein gene. Variability in the intensity of the bands in the transformants is due to the variation in amount of DNA used, efficiency of restriction digestion, and possibly due to copy number, although copy number was not determined. The background at the top of the lanes is the result of undigested DNA and background due to large amount of DNA present. Variation in the amount of DNA used is due to the limits of quantification using gel electrophoresis of a small quantity of the isolated DNA. Sensitivity could be increased by using a higher percentage of the isolated DNA, but would compromise the amount available for the Southern hybridization. Spectrophotometry for quantifying the DNA was found to be more variable than gel electrophoresis. Southern analysis was successfully repeated for all samples shown.

Western Analysis

Western analysis of transformants can be difficult because transformed plants often express the protein in very low quantities. Initial experiments were performed using 12 μl of sample removed from a stock supply of approximately 1 cm^2 of leaf material in 1000 μl of protein

extraction buffer. No coat protein was detected in the transformants although the coat protein could be detected in control plants inoculated with CTV isolates T30 or T36. Western analysis was performed on the 'Nova' tangelo plants as described in the materials and methods. On one occasion a very weak band was detected in N32 and N34 but not in N33, but this was not repeatable.

It is often necessary to concentrate low abundance proteins in order to detect them on a western blot. An experiment was performed to test whether the coat protein gene could be detected after concentration using a Jouan RC10 Concentrator/Evaporator. Duplicate samples were isolated from the three 'Nova' tangelo plants, plus a healthy and CTV infected control. One set of samples was boiled prior to concentration while the other set was not. The samples were then concentrated to approximately 1/5 volume. Prior to loading the polyacrylamide gel both sets of samples were then boiled and the western blotting performed as described in the materials and methods. The antibody used for detection was the polyclonal 1053 antibody provided by Dr. R. F. Lee. The results using the samples that were boiled prior to concentration were all negative. However, the results from the samples which were not boiled were as expected and are shown in figure 4-8.

Shortly after achieving the above results the Jouan RC10 concentrator/evaporator suffered a major breakdown, so a new way of increasing the protein concentration in the samples for western hybridizations. The same amount of leaf material (approx. 10 cm^2) was ground as before but resuspended in 700 μl of protein extraction buffer. Instead of using 12 μl of sample, 30 μl of sample was used. The exception

Figure 4-8. Western immunoblot of the 'Nova' tangelo transformants. Samples were prepared as described in the text. Lane 1 is transformant N32, lane 2 is transformant N33 , lane 4 is transformant N34, lane 4 is a T30 infected plant and lane 5 is a non-transformed 'Nova' tangelo control.

was the T36 or T30 positive controls for which only 5 μl of sample was used. Also, the antibodies were diluted at 1:20000 v/v in order to decrease the background (R.F. Lee, pers. comm.). The primary antibody used for 'Nova' tangelo was G604 (from M. Keremane) and for 'Rohde Red Valencia' sweet orange the primary antibody used was MCA13. G604 will detect isolate T30 while MCA13 is a monoclonal antibody specific to decline isolates, including T36. The results for the 'Nova' tangelo are shown in figure 4-9, and the results for 'Rohde Red Valencia' sweet orange are shown in figure 4-10. Migration of the proteins in the transformant samples was slowed because of the amount of sample loaded. Similarly, partly due to the large amount of protein loaded, there was a significant background for the high molecular weight, abundant proteins. These are evident in the upper portion of the blots. Confirmation that these bands are associated with the coat protein can be inferred based on the lack of these bands in the non-infected controls. However, the quality of the western blots suggests that the protein may be expressed in quantities too low to be detected by western immunoblot analysis, and no definite confirmation of protein expression can be made based on these results. It is possible that proteins may be detectable using procedures to seperate the proteins, but these experiments were not conducted.

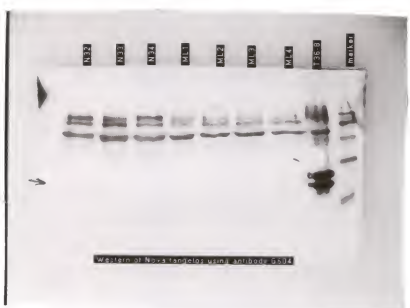
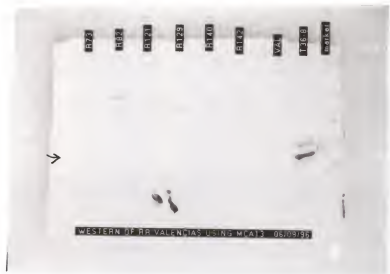


Figure 4-9. Western immunoblot of transformed 'Nova' tangelo using primary antibody G604. Lanes 1-3 -- transformants N32, N33, N34 respectively. lanes 4-7 -- non infected, non transformed Mexican Lime plants, lane 8 -- a T30 infected control plant, and lane 9 -- molecular weight markers. Arrow points to the band corresponding to the coat protein. The Lane marked T36-8 is mislabeled and should be T30.



a). lane 1 -- R73, lane 2 -- R82, lane 3 -- R121, lane 4 -- R129, lane 5 -- R140, lane 6 -- R142, lane 7 -- 'valencia' nontransformed control, lane 8 -- a T36 infected plant, lane 9 -- molecular weight markers.

b). lane 1 -- R146, lane 2 -- R147, lane 3 -- R150, lane 4 -- R152, lane 5 -- R269, lane 6 -- R270, lane 7 -- 'Valencia' nontransformed control, lane 8 -- T36 infected plant and lane 9 -- molecular weight markers.

Figure 4-10. Western immunoblot analysis of the 'Rhode Red Valencia' sweet orange transgenic plants using MCA13 antibody.

c). lane 1 -- R83, lane 2 -- R124, lane 3 -- R149, lane 4 -- R153, lane 5 -- R268, lane 7 -- 'valencia' nontransformed control, lane 8 -- T36 infected plant, lane 9 -- molecular weight markers.

d). lane 1 -- R271, lane 2 -- R271b, lane 3 -- R272, lane 4 -- R276, lane 5 -- R328, lane 7 -- 'Valencia' nontransformed control, lane 8 -- T36 infected plant, and lane 9 -- molecular weight markers.

Figure 4-10--continued.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The successful transformation of Citrus scions with the coat protein gene of Citrus Tristeza Virus using PEG-mediated direct DNA uptake was achieved. Three 'Nova' tangelo transformed with the coat protein gene from mild isolate T30 (from 59 total regenerants), and 22 'Rohde Red Valencia' sweet orange transformed with the coat protein gene from florida severe isolate T36 (from 500 total regenerants) were obtained. No transformants were obtained from the 12 'Valencia' sweet orange regenerants. It is important to note that these transgenic plants were obtained without selection at the cellular level. Identification was made using the polymerase chain reaction for the 'Nova' tangelos, and Dot Blot hybridization for the 'Rohde Red Valencia' sweet orange plants. The time required to select after regeneration of plants was not significantly different than the time required for selection at the cellular level. Confirmation of stable transformation was shown by Southern hybridization and western analysis. Western analysis suggested a low level of expression of the coat protein gene in the transgenic plants.

The results also showed that pooled PCR can be used for selection of transgenics, as was demonstrated for the 'Nova' tangelos, but PCR is also very susceptible to outside interference. In order to reliably use PCR for screening, measures need to be taken to guard against contamination

from outside sources. Screening by pooled Southern dot blot hybridization can also be used successfully, as was demonstrated with the 'Rohde Red Valencia' sweet orange. Southern dot blot hybridization was found to be as easy and fast as PCR, and by using the DNA isolation procedure that was used for later experiments, efficiency could be even better. These results are summarized in Figure 5-1.

Evidence of somaclonal variation was difficult to ascertain. There were differences in growth rate, particularly for the 'Rohde Red Valencia' sweet orange, but the reasons for this are difficult to determine. The stunting of the plants could be due to a positional effect of the inserted gene, or the effect of the coat protein expression, or a combination of these, and a number of other unknown effects.

The regenerated transgenic plants are now ready to be tested by challenge inoculation with different severe quick decline and stem-pitting isolates of CTV in the greenhouse. The results of these tests will help to determine whether these plants have acquired a greater level of resistance against infection by CTV. If these tests are positive, field testing can then be carried out. Selection will indirectly be made at this point for other important horticultural traits, and thus for the possible somaclonal variants exhibiting normal or improved horticultural traits. Positive results in the field will mean that tristeza resistant *Citrus* scions have been achieved.

PLANT #	APPEARANCE	SOUTHERN POSITIVE	WESTERN POSITIVE
N32	Normal	yes	yes
N33	Normal	yes	yes
N34	Normal	yes	yes
R73	somewhat small plant	No	yes
R82	Small, w/ elongate leaves	no	yes
R83	Slow growing	no	yes
R121	Normal	yes	yes
R124	Normal	yes	yes
R129	Normal	yes	yes
R140	Normal	yes	yes
R142	Leaves slightly smaller	no	yes
R146	Normal	yes	yes
R147	Normal	no	yes
R149	Very small plant	no	yes
R150	Normal	yes	yes
R152	Normal	yes	yes
R153	Normal	yes	yes
R268	somewhat small plant	yes	yes
R269	Normal	yes	yes
R270	Normal	???	yes
R271	Normal	yes	yes
R271b	Normal	yes	yes
R272	Normal	yes	yes
R276	Normal	yes	yes
R328	Normal	yes	yes

Table 5-1. Summary of the transgenic plants regenerated in this experiment, and whether confirmed by Southern and/or Western analysis.

APPENDIX A

TISSUE CULTURE AND PROTOPLAST MEDIAS

MT BASAL MEDIUM (Murashige and Tucker, 1969)

MT MACRO STOCK	STOCK CONC.	FINAL CONC.
NH_4NO_3	165 g/L	1.65 g/L
KNO_3	190 g/L	1.90 g/L
$MgSO_4 * 7H_2O$	37 g/L	0.37 g/L
KH_2SO_4	17 g/L	0.17 g/L

MT MICRO STOCK	STOCK CONC.	FINAL CONC.
H_3BO_3	0.6200 g/L	0.0062 g/L
$MnSO_4 * H_2O$	1.6800 g/L	0.0168 g/L
$ZnSO_4 * 7H_2O$	0.8600 g/L	0.0086 g/L
KI	0.0830 g/L	0.00083 g/L
$Na_2MoO_4 * 2H_2O$	0.0250 g/L	0.00025 g/L
$CuSO_4 * 5H_2O$	0.0025 g/L	0.000025 g/L
$CoCl_2 * 6H_2O$	0.0025 g L	0.000025 g/L

MT IRON STOCK	STOCK CONC.	FINAL CONC.
Na_2EDTA	7.45 g/L	0.03725 g/L
$FeSO_4 * 7H_2O$	5.57 g/L	0.02785 g/L

MT CALCIUM STOCK	STOCK CONC.	FINAL CONC.
$CaCl_2 * 2H_2O$	29.33 g/L	0.4400 g/L
MT VITAMIN	STOCK CONC.	FINAL CONC.
myo-Inositol	10 g/L	0.1 g/L
Thiamine-HCl	1 g/L	0.01 g/L
Pyridoxine-HCl	1 g/L	0.01 g/L
Nicotinic Acid	0.5 g/L	0.005 g/L
Glycine	0.2 g/L	0.002 g/L

EME (Grosser and Gmitter, 1990)

MT Macro Stock	10 ml/L
MT Micro Stock	10 ml/L
MT Vitamin Stock	10 ml/L
MT Calcium Stock	15 ml/L
MT Iron Stock	5 ml/L
Sucrose	50 g/L
Malt Extract	0.5 g/L

Adjust pH to 5.8 with KOH
Add 8.0 g/L Agar and Autoclave 15 minutes at 15 psi.
Note: for suspension culture media omit the agar.

1500 (Grosser and Gmitter, 1990)

1500 media is exactly the same as EME with the exception that the malt extract concentration is increased to 1.500 g/L.

1/2-1/2 (Grosser and Gmitter, 1990)

MT Macro Stock	5 ml/L
BH3 Macro Stock ***	5 ml/L
MT Micro Stock	10 ml/L
MT Vitamin Stock	10 ml/L
MT Calcium Stock	15 ml/L
MT Iron Stock	5 ml/L
L(+) Glutamine	1.55 g/L
Malt Extract	0.5 g/L
Sucrose	50 g/L

Adjust pH to 5.8 with KOH
For solid media add 8.0 g/L Agar
Autoclave at 15 psi for 15 minutes

*** See BH3 Stocks later in this appendix for BH3 Macro Stock.

B+ (Grosser and Gmitter, 1990)

MT Macro Stock	10 ml/L
MT Micro Stock	10 ml/L
MT Vitamin Stock	10 ml/L
MT Calcium Stock	15 ml/L
MT Iron Stock	5 ml/L
Sucrose	25 g/L
GA3	1 mg/L
Coumarin	100 uM/L
NAA	0.02 mg/L
Coconut Water	20 ml/L

Adjust pH to 5.8 with KOHAdd 8.0 g/L agar and autoclave for 15 minutes at 15 psi.

ROOTING MEDIUM (Grosser and Gmitter, 1990)

MT Macro Stock	5 ml/L
MT Micro Stock	5 ml/L
MT Vitamin Stock	5 ml/L
MT Calcium Stock	15 ml/L
MT Iron Stock	5 ml/L
NAA	0.02 mg/L
Sucrose	25 g/L
Activated Charcoal	0.5 g/L

Adjust pH to 5.8 with KOH

Add 8.0 g/L agar, put approx. 100 ml per magenta boxes and autoclave.

BH3 Stock Solutions (Kao and Michayluk, 1975, Grosser and Gmitter, 1990)

BH3 MACRO STOCK	STOCK CONC.	FINAL CONC.
$MgSO_4$	37 g/L	0.37 g/L
KH_2PO_4	17 g/L	0.17 g/L
KCl	150 g/L	1.50 g/L

BH3 VITAMIN STOCK A	STOCK CONC.	FINAL CONC.
Calcium Pantothenate	0.050 g/100 ml	1.0 mg/L
Ascorbic Acid	0.100 g/100 ml	2.0 mg/L
Choline Chloride	0.050 g/100 ml	1.0 mg/L
p-Aminobenzoic Acid	0.001 g/100 ml	0.02 mg/L
Folic Acid	0.020 g/100 ml	0.40 mg/L
Riboflavin	0.010 g/100 ml	0.20 mg/L
Biotin	0.001 g/100 ml	0.02 mg/L

BH3 VITAMIN STOCK B	STOCK CONC.	FINAL CONC.
Vitamin A (retinol)	0.001 g/100 ml	.01 mg/L
Vitamin D_3 (cholecalciferol)	0.001 g/100 ml	.01 mg/L
Vitamin B_{12}	0.002 g/100 ml	.02 mg/L

BH3 KI STOCK	STOCK CONC.	FINAL CONC.
KI	.075 g/100 ml	0.75 mg/L

BH3 SUGAR ALCOHOL STOCK	STOCK CONC.	FINAL CONC.
Fructose	2.5 g/100 ml	250 mg/L
Ribose	2.5 g/100 ml	250 mg/L
Xylose	2.5 g/100 ml	250 mg/L
Mannose	2.5 g/100 ml	250 mg/L
Rhamnose	2.5 g/100 ml	250 mg/L
Cellobiose	2.5 g/100 ml	250 mg/L
Galactose	2.5 g/100 ml	250 mg/L

BH3 ORGANIC ACID STOCK	STOCK CONC.	FINAL CONC.
Sodium Pyruvate	0.2 g/100 ml	20 mg/L
Citric Acid	0.4 g/100 ml	40 mg/L
Malic Acid	0.4 g/100 ml	40 mg/L
Fumaric Acid	0.4 g/100 ml	40 mg/L

BH3 Media (Grosser and Gmitter, 1990)

BH3 Macro Stock	10 ml/L
MT Micro Stock	10 ml/L
MT Vitamin Stock	10 ml/L
MT Calcium Stock	15 ml/L
MT Iron Stock	5 ml/L
BH3 Vitamin Stock A	2.0 ml/L
BH3 Vitamin Stock B	1.0 ml/L
BH3 KI Stock	1.0 ml/L
BH3 Sugar Alcohol Stock	10 ml/L
BH3 Organic Acid Stock	10 ml/L
Coconut Water	20 ml/L
Malt Extract	1.0 g/L
Casein Acid Hydrolysate	0.25 g/L
Glutamine	3.1 g/L
Sucrose	51.35 g/L
Mannitol	82.00 g/L

Adjust pH to 5.7 with KOH
Filter sterilize using .22 micron filter units.
Decant into sterile bottles and store in the dark.

CPW Salts (Frearson et al., 1973)

STOCK 1	STOCK CONC.	FINAL CONC.
KH_2PO_4	0.272 g/100 ml	27.2 mg/L
KNO_3	1.000 g/100 ml	100.0 mg/L
$MgSO_4$	2.500 g/100 ml	250.0 mg/L
KI	0.002 g/100 ml	0.200 mg/L

$CuSO_4$	0.00003 g/100ml	0.0003 mg/L

STOCK 2	STOCK CONC.	FINAL CONC.
$CaCl_2$	1.50 g/100 ml	150 mg/L

Add 1 ml/100ml of each stock. Adjust pH to 5.8. filter sterilize through a .22 micron filter. Store in cold.

For CPW13MAN add 13 g/100 ml of mannitol

For CPW25SUC add 25 g/100 ml of sucrose

store these in cold also.

ENZYME SOLUTION (Grosser and Gmitter, 1990)

Mannitol (0.7 M)	5.12 g/40 ml
$CaCl_2$ (24.5 mM)	0.144 g/40 ml
MES (6.15 mM)	0.048 g/40 ml
NaH_2PO_4 (.92 mM)	0.044 g/40 ml
1% Cellulase (Onuzuka RS)	0.4 g/40 ml
1% Macerase	0.4 g/40 ml
0.2% Pectolyase Y 23	0.08 g/40 ml

Adjust pH to 5.6 and filter sterilize using .22 micron filter.

FUSION SOLUTIONS (Grosser and Gmitter, 1990)

PEG SOLUTION

Polyethylene Glycol (MW 1500 from Aldrich)	40 g/100ml
$CaCl_2 * 2H_2O$	0.97 g/100 ml
Glucose	5.41 g/100 ml

Adjust pH to 6.0 with KOH over a period of 1-2 hours.
Filter sterilize with a .22 micron filter.

SOLUTION A

Glucose	7.21 g/100 ml
$CaCl_2 * 2H_2O$	0.97 g/100 ml
DMSO	10 ml/100 ml

Adjust pH to 6.0 with KOH. Filter sterilize with a .22 micron filter.

SOLUTION B

Glycine	2.25 g/100 ml

Adjust pH to 10.5 with KOH. Filter sterilize with a .22 micron filter.

APPENDIX B

MOLECULAR BIOLOGY BUFFERS (Sambrook et al., 1989)

5X TBE

Tris	54 g/L
Boric Acid	27.5 g/L
0.5 M EDTA	20 ml/L

TE

Tris	1.21 g/L
0.5 M EDTA	2 ml/L

Adjust pH to 8.0 with 12 M HCl

20 X SSC

NaCl	175.3 g/L
Sodium Citrate	88.2 g/L

Adjust pH to 7.0

LB Medium (Luria-Bertani Medium)

Bacto-tryptone	10 g/L
Bacto-yeast extract	5 g/L
NaCL	10g/L

Adjust pH to 7.0 with NaOH. For solid medium add 15 g/L of Agar. Autoclave for 20 minutes at 15 psi.

2 X YT Medium

bacto-tryptone	16 g/L
bacto-yeast extract	10 g/L
NaCl	5 g/L

Adjust pH to 7.0 with NaOH. Autoclave for 20 minutes at 15 psi.

Potassium Acetate Solution

5M Potassium Acetate	60 ml/ 100 ml
Glacial Acetic Acid	11.5 ml/ 100 ml
dd H_2O	28.5 ml/ 100 ml

SucTET

Sucrose	8.8 g/ 100 ml
1 M Tris-HCl, pH 8.0	1.0 ml/ 100 ml
0.5 M EDTA, pH 8.0	10 ml/ 100 ml
Triton X100	500 µl/ 100 ml

DNA Extraction Buffer

1 M Tris-HCL, pH 8.0	100 ml/ L
0.5 M NaEDTA, pH 8.0	100 ml/ L
NaCl	29.22 g/ L

Add 700 µl/L of 2-mercaptoethanol and 2 ml of SDS for each 15 ml of DNA Extraction Buffer just prior to use and incubate at 65 C to make sure SDS is in solution.

Stocks for 14% Polyacrylamide Gels

STACKING GEL STOCK for 75 ml

H_2O	44.8 ml
Tris-HCl, pH 6.8	20.0 ml
Acryl-Bis, 40%	7.7 ml
10% SDS	0.75 ml

14% RUNNING GEL STOCK for 50 ml

H_2O	18.6 ml
Acryl-Bis, 40%	17.5 ml
Tris-HCl, pH 9.3	13.4 ml
10% SDS	0.5 ml

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BIOGRAPHICAL SKETCH

Jay Schell was born July 4, 1962, in Bowman, ND. For seven years he lived in nearby Reeder, ND, before moving to Boulder, CO, in 1969. He attended second grade through high school in Boulder, graduating from Fairview High School in 1980. He then attended Colorado State University, where he received his Bachelor of Science degree in botany in 1984.

Upon receiving his B.S. degree he moved to Salt Lake City, UT for three years, where he was employed by Native Plants, Inc. (NPI). From there he moved to Florida in 1988 to attend the University of Florida. He received his Master of Science degree in horticulture from the University of Florida in 1991. He is an avid sports fan spending much of his free time participating in tennis, basketball, golf and soccer. He hopes to become a frequent participant in tennis tournaments upon starting his science career, and possibly a part time coaching career. He has accepted a postdoctoral position with the Plant Science Department at South Dakota State University.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a disseration for the degree of Doctor of Philosophy.

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